Metabarcoding and Metagenomics 7: 51–72
DOI 10.3897/mbmg.7.94298

Research Article

# Development of two new sets of PCR primers for eDNA metabarcoding of brittle stars (Echinodermata, Ophiuroidea)

**Masanori Okanishi**[1,2], **Hisanori Kohtsuka**[1], **Qianqian Wu**[3], **Junpei Shinji**[1,4,5], **Naoki Shibata**[6], **Takashi Tamada**[6], **Tomoyuki Nakano**[7], **Toshifumi Minamoto**[3]

1 *Misaki Marine Biological Station, The University of Tokyo, 1024 Koajiro, Miura, Kanagawa 238-0225, Japan*
2 *Faculty of Human Environmental Studies, Hiroshima Shudo University, 1-1-1 Ozuka-Higashi, Asaminami-ku, Hiroshima 731-3195, Japan*
3 *Graduate School of Human Development and Environment, Kobe University, 3-11 Tsurukabuto, Nada, Kobe, Hyogo 657-8501, Japan*
4 *Center for Ocean Literacy and Education, The University of Tokyo, 2-11-16 Yayoi, Bunkyo, Tokyo 113-0032, Japan*
5 *Regional Fish Institute, Ltd., Yoshida-Honmachi, Sakyo-ku, Kyoto-shi, Kyoto, 606-8501, Japan*
6 *Environmental Research & Solutions Co. Ltd., 2-3-9 Hikaridai, Seika, Soraku, Kyoto 619-0237, Japan*
7 *Seto Merine Biological Laboratory, Kyoto University, 459 Shirahama, Nishimuro, Wakayama 649-2211, Japan*

Corresponding author: Masanori Okanishi (okahoku@gmail.com)

**Academic editor:** Filipe Costa | **Received** 31 August 2022 | **Accepted** 26 October 2022 | **Published** 28 February 2023

## Abstract

Brittle stars (class Ophiuroidea) are marine invertebrates comprising approximately 2,100 extant species, and are considered to constitute the most diverse taxon of the phylum Echinodermata. As a non-invasive method for monitoring biodiversity, we developed two new sets of PCR primers for metabarcoding environmental DNA (eDNA) from brittle stars. The new primer sets were designed to amplify 2 short regions of the mitochondrial 16S rRNA gene, comprising a conserved region (111–115 bp, 112 bp on average; named "16SOph1") and a hyper-variable region (180–195 bp, 185 bp on average; named "16SOph2") displaying interspecific variation. The performance of the primers was tested using eDNA obtained from two sources: a) rearing water of an 2.5 or 170 L aquarium tanks containing 15 brittle star species and b) from natural seawater collected around Misaki, the Pacific coast of central Japan, at depths ranging from shallow (2 m) to deep (> 200 m) sea. To build a reference library, we obtained 16S rRNA sequences of brittle star specimens collected from around Misaki and from similar depths in Japan, and sequences registered in International Nucleotide Sequence Database Collaboration. As a result of comparison of the obtained eDNA sequences with the reference library 37 (including cryptic species) and 26 brittle star species were detected with certain identities by 16SOph1 and 16SOph2 analyses, respectively. In shallow water, the number of species and reads other than the brittle stars detected with 16SOph1 was less than 10% of the total number. On the other hand, the number of brittle star species and reads detected with 16SOph2 was less than half of the total number, and the number of detected non-brittle star metazoan species ranged from 20 to 46 species across 6 to 8 phyla (only the reads at the "Tank" were less than 0.001%). The number of non-brittle star species and reads at 80 m was less than 10% with both of the primer sets. These findings suggest that 16SOph1 is specific to the brittle star and 16SOph2 is suitable for a variety of marine metazoans. It appears, however, that further optimization of primer sequences would still be necessary to avoid possible PCR dropouts from eDNA extracts. Moreover, a detailed elucidation of the brittle star fauna in the examined area, and the accurate identification of brittle star species in the current DNA databank is required.

## Key Words

16SOph1, 16SOph2, deep sea waters, environmental monitoring, mitochondrial 16S rRNA, Sagami Bay

## Introduction

Classical methods of biodiversity monitoring have been primarily based on the collection of specimens and subsequent morphology-based identification. Such biodiversity monitoring is costly and time-consuming, and requires considerable expertise for various taxonomic groups. Recent technological developments in molecular ecology have provided a novel tool for species detection using DNA present in aquatic or terrestrial environments ("environmental

Copyright *Masanori Okanishi et al.* This is an open access article distributed under the terms of the Creative Commons Attribution License (CC BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

DNA" or "eDNA") (Taberlet et al. 2012). There are 2 major approaches, a species-specific approach (eDNA barcoding) and a multi-species approach (eDNA metabarcoding) (Komai et al. 2019). The latter approach has been developed with rapidly developed high-throughput next-generation sequencing (NGS) (e.g. Thomsen et al. 2012; Miya et al. 2015; Valentini et al. 2016). The application of eDNA is now increasing in studies of biodiversity, aquatic ecology and conservation biology (Bohmann et al. 2014; Díaz-Ferguson and Moyer 2014). In particular, with regard to aquatic environments, the multi-species assessment and monitoring of fauna using eDNA have focused mainly on vertebrates (e.g. Thomsen et al. 2012; Kelly et al. 2014; Miya et al. 2015; Andruszkiewicz et al. 2017; Ushio et al. 2017, 2018), which are known to release abundant DNA derived from faeces, body mucus, blood and sloughed tissue or scales (Bohmann et al. 2014). The applicability of eDNA metabarcoding for detecting aquatic invertebrates has received growing attention (Thomsen et al. 2012; Rees et al. 2014; Gielings et al. 2021; Madduppa et al. 2021), but in Japan the only reported application was for detecting decapod crustacean biodiversity (Komai et al. 2019). The vast majority of previous studies on crustaceans yielded a "species-specific" approach detecting invasive species (Komai et al. 2019) or monitoring seasonal migrations of particular species (Wu et al. 2018). The deep sea (depth deeper than 200 m; e.g. Gage and Tyler 1991) accounts for more than 90% of the volume of the marine environment and a large part of the biosphere of the globe (e.g. Fujikura et al. 2012). Therefore, it is very important to establish a method for monitoring organisms in the ocean, including the deep sea, in order to assess the global environment. At least in Japan, however, there have been no examples of eDNA metabarcoding analysis of environmental water in the deep sea.

Brittle stars (Ophiuroidea: Echinodermata) are the most diverse taxon of echinoderms, comprising approximately 2,100 extant species (Stöhr et al. 2012). They occur on diverse benthic substrates in every ocean, distributed from polar regions through to the Equator, ranging from shallow intertidal to abyssal zones down to 8,105 m (Stöhr et al. 2012). Morphological and behavioral specializations in ophiuroids, which are mainly represented by their flexible arms, enable them to live in a wide range of habitats: under rocks; in interstices within sponges and hard corals; on muddy bottoms, where some species can be found in aggregates; infaunally buried in sediments, with arms extended out of the bottom for filter feeding; and on the surface of various animals such as octocorals and hydrocorals (e.g. Okanishi 2016). Therefore, they are very useful as target organisms for marine environmental metabarcoding because the DNA of brittle stars, which have large populations and inhabit various environments, is likely to remain in marine environmental waters.

The selection of a marker is important in eDNA metabarcoding (Coissac et al. 2012; Deagle et al. 2014). The hyper-variable region of mitochondrial 12S rRNA has been developed as a useful marker in fish metabarcoding (Miya et al. 2015), and the conserved region of 16S rRNA has been developed as a marker in decapod crustaceans metabarcoding (Komai et al. 2019). In this study, we developed 2 primer sets for the 16S rRNA region, which is often used for molecular phylogenetic studies in brittle stars and contains both hypervariable and conserved regions, for assessment and monitoring of brittle star's biodiversity (e.g. Okanishi and Fujita 2013; Glynn et al. 2020). These primer sets were used to detect brittle star species in seawater eDNA collected from around the Misaki Marine Biological Station (**MMBS**: The University of Tokyo, Kanagawa Prefecture, Japan), from the shallow to the deep sea area, as well as in the rearing water of aquarium tanks in MMBS where brittle star species were kept. In addition, we tested the detection ability of the new primer sets by comparing it with the sequences of 59 species collected from the related environment.

# Materials and methods

## Design of new primer sets

Generally, the mitochondrial 16S rRNA and COI genes reflects the species diversity of animals, and COI gene has a faster base substitution rate than 16S (e.g. Palumbi 1996; Deagle et al. 2014). In brittle star, however, there is often little difference in the results of molecular phylogenetic analyses using these two regions (Boissin et al. 2008; Hunter and Halanych 2008; Cho and Shank 2010; Okanishi et al. 2018). Furthermore, since the COI gene is a coding region, many mutations accumulate in the third codon, making it relatively difficult to create universal primers to detect many species. Therefore, in this study, the mitochondrial 16S rRNA gene was selected as the target sequence for eDNA metabarcoding. This gene contains both hyper-variable and conserved regions (Gray et al. 1984; Okanishi and Fujita 2013). In order to select suitable regions of 2 primer sets for species detection by metabarcoding analysis, we referred to 1,839 16S rDNA sequences from 132 brittle star species obtained from the International Nucleotide Sequences Database (INSD). The number of species and length of the sequences were 32 with 118–160 bp for 16SOph1, and were 119 with 160–200 bp for 16SOph2 (Table 1). For developing 16SOph2, we newly sequenced partial sequences of 16S rRNA of 13 brittle star species by the same method with constructing custom reference data base (Table 1; see also "Determination of target sequences of specimens collected from the study area" below). These sequences were from species across 6 major orders and 18 superfamilies of brittle stars (Table 1). The amplified products were designed to be less than 200 bp in length, taking into account the fact that DNA in environmental water is short and fragmented (Riaz et al. 2011; Miya et al. 2015; Valentini et al. 2016). The downloaded and newly obtained sequences were aligned using Clustal W (Thompson et al. 1994) implemented in MEGA7 (Kumar et al. 2016) with a default set of parameters. In

**Table 1.** Sequences used to design Oph16S1 and Oph16S2 primers. Scientific names follow those as registered in INSD. Species with an asterisk after their specific names indicate that their 16S rRNA sequences were newly sequenced in this study.

| Superorder | Order | Family | Species | 16SOph1 | Accession number | 16SOph2 | Accession number |
|---|---|---|---|---|---|---|---|
| Euryophiurida | Euryalida | Asteronychidae | *Asteronyx loveni* | ○ | LC276323 | ○ | AB605076.1 |
| | | | *Asteronyx reticulata* | | - | ○ | LC276294.1 |
| | | | *Asteronyx longifissus* | | - | ○ | KM014337.1 |
| | | Euryalidae | *Asteromorpha capensis* | | - | ○ | AB758510.1 |
| | | | *Asteromorpha rousseaui* | | - | ○ | AB758509.1 |
| | | | *Asteroschema ajax* | | - | ○ | AB605078.1 |
| | | | *Asteroschema clavigerum* | | - | ○ | HM587842.1 |
| | | | *Asteroschema edmondsoni* | | - | ○ | AB758486.1 |
| | | | *Asteroschema ferox* | ○ | AB605079.1 | ○ | AB605079.1 |
| | | | *Asteroschema horridum* | | - | ○ | AB758487.1 |
| | | | *Asteroschema intectum* | | - | ○ | AB758484.1 |
| | | | *Asteroschema migrator* | | - | ○ | AB758485.1 |
| | | | *Asteroschema oligactes* | | - | ○ | AB758483.1 |
| | | | *Asteroschema salix* | | - | ○ | AB758482.1 |
| | | | *Asterostegus maini* | | - | ○ | AB758507.1 |
| | | | *Asterostegus tuberculatus* | | - | ○ | AB758515.1 |
| | | | *Astrobrachion adhaerens* | | - | ○ | AB605081.1 |
| | | | *Astrobrachion constrictum* | | - | ○ | AB605082.1 |
| | | | *Astroceras annulatum* | ○ | AB605089.1 | ○ | AB605089.1 |
| | | | *Astroceras aurantiacum* | | - | ○ | AB758513.1 |
| | | | *Astroceras compar* | | - | ○ | AB605090.1 |
| | | | *Astroceras nodosum* | | - | ○ | AB758506.1 |
| | | | *Astroceras pergamenum* | ○ | AB605091.1 | ○ | AB605091.1 |
| | | | *Astroceras pleiades* | | - | ○ | AB605708.1 |
| | | | *Astroceras spinigerum* | | - | ○ | AB758508.1 |
| | | | *Astrocharis monospinosa* | ○ | AB605083.1 | | - |
| | | | *Euryale aspera* | | - | ○ | AB605093.1 |
| | | | *Ophiocreas caudatus* | ○ | AB605085.1 | | - |
| | | | *Ophiocreas glutinosum* | ○ | AB605086.1 | | - |
| | | | *Ophiocreas japonicus* | ○ | AB758488.1 | | - |
| | | | *Sthenocephalus anopla* | | - | ○ | AB605094.1 |
| | | | *Trichaster acanthifer* | | - | ○ | AB605095.1 |
| | | | *Trichaster flagellifer** | | - | ○ | O288 |
| | | | *Trichaster palmiferus* | | - | ○ | AB605096.1 |
| | | Gorgonocephalidae | *Asteroporpa australiensis* | | - | ○ | AB605098.1 |
| | | | *Asteroporpa hadracantha* | ○ | AB605097.1 | ○ | AB605097.1 |
| | | | *Asteroporpa reticulata* | | - | ○ | AB605099.1 |
| | | | *Asteroporpa muricatopatella* | | - | ○ | AB605100.1 |
| | | | *Astroboa arctos* | ○ | AB605101.1 | ○ | AB605101.1 |
| | | | *Astroboa globifera* | ○ | AB605102.1 | ○ | AB605102.1 |
| | | | *Astroboa nuda* | | - | ○ | AB758499.1 |
| | | | *Astroboa nigrofurcata* | | - | ○ | AB758505.1 |
| | | | *Astrochele pacifica* | | - | ○ | AB605104.1 |
| | | | *Astrochele lymani* | | - | ○ | AB758504.1 |
| | | | *Astrochlamys sol* | | - | ○ | AB758503.1 |
| | | | *Astrocladus coniferus* | ○ | AB605105.1 | ○ | AB605105.1 |
| | | | *Astrocladus exiguus* | | - | ○ | AB605106.1 |
| | | | *Astroclon suensoni* | | - | ○ | LC272070.1 |
| | | | *Astroclon propugnatoris* | | - | ○ | AB605108.1 |
| | | | *Astrocrius* sp. | ○ | AB605107.1 | | - |
| | | | *Astrodendrum sagaminum* | ○ | AB605109.1 | ○ | AB605109.1 |
| | | | *Astroglymma sculptum* | | - | ○ | AB605111.1 |
| | | | *Astroglymma sculpta** | | - | ○ | O289 |
| | | | *Astrohamma tuberculatum* | | - | ○ | AB605112.1 |
| | | | *Astrotoma agassizii* | | - | ○ | AB758493.1 |
| | | | *Astrotoma drachi* | | - | ○ | AB758494.1 |
| | | | *Astrothorax misakiensis* | ○ | AB605116 | | - |
| | | | *Conocladus australis* | | - | ○ | AB758491.1 |
| | | | *Gorgonocephalus chilensis* | | - | ○ | AB758495.1 |
| | | | *Gorgonocephalus eucnemis* | ○ | AB605121.1 | ○ | AB605121.1 |
| | | | *Gorgonocephalus pustulatum* | | - | ○ | AB605122.1 |
| | | | *Gorgonocephalus tuberosus* | ○ | AB758496.1 | ○ | AB758496.1 |
| Ophintegrida | Ophiurida | Ophiuridae | *Ophiocten megaloplax* | | - | ○ | KF713454.1 |
| | | | *Ophionotus victoriae* | | - | ○ | FJ917294.1 |
| | | | *Ophiura albida* | | - | ○ | AY652507.1 |
| | | | *Ophiura kinbergi** | ○ | MH910618.1 | ○ | eo-03 |
| | | | *Ophiura ooplax** | | - | ○ | eo-04 |
| | | | *Ophiura ophiura* | | - | ○ | AY652508.1 |
| | | | *Ophiura sarsii* | ○ | MH780492.1 | | - |
| | | Ophiopyrgidae | *Ophioplinthus gelida* | | - | ○ | GU226981.1 |
| | | Ophiosphalmidae | *Ophiomusium* cf. *glabrum* | ○ | KU519519.1 | | - |

| Superorder | Order | Family | Species | 16SOph1 | Accession number | 16SOph2 | Accession number |
|---|---|---|---|---|---|---|---|
| Ophintegrida | Amphilepidida | Ophiactidae | *Ophiactis lymani* | ○ | KP128039.1 | ○ | KM234226.1 |
| | | | *Ophiactis rubropoda** | | - | ○ | eo-09 |
| | | Ophiopholidae | *Ophiopholis aculeata* | | - | ○ | AY652513.1 |
| | | | *Ophiopholis japonica* | ○ | MK343095.1 | ○ | HM473898.1 |
| | | | *Ophiopholis mirabilis* | ○ | MK343098.1 | | |
| | | Ophiotrichidae | *Macrophiothrix belli* | | - | ○ | AH013198.2 |
| | | | *Macrophiothrix caenosa* | | - | ○ | AH013199.2 |
| | | | *Macrophiothrix demessa* | | - | ○ | AH013200.2 |
| | | | *Macrophiothrix koehleri* | | - | ○ | AY365153.1 |
| | | | *Macrophiothrix lampra* | | - | ○ | AY365154.1 |
| | | | *Macrophiothrix leucosticha* | | - | ○ | AH013202.2 |
| | | | *Macrophiothrix longipeda* | ○ | AY365160.1 | ○ | AH013203.2 |
| | | | *Macrophiothrix lorioli* | | - | ○ | AH013204.2 |
| | | | *Macrophiothrix megapoma* | | - | ○ | AH013205.2 |
| | | | *Macrophiothrix nereidina* | ○ | AY365167.1 | ○ | AY365169.1 |
| | | | *Macrophiothrix paucispina* | | - | ○ | AY365170.1 |
| | | | *Macrophiothrix rhabdota* | | - | ○ | AH013209.2 |
| | | | *Macrophiothrix robillardi* | | - | ○ | AY365176.1 |
| | | | *Ophiomaza cacaotica** | | - | ○ | eo-12 |
| | | | *Ophiothela danae** | | - | ○ | eo-25 |
| | | | *Ophiothrix exhibita* | | - | ○ | eo-13 |
| | | | *Ophiothrix angulata* | | - | ○ | MH281603.1 |
| | | | *Ophiothrix caespitosa* | | - | ○ | AH013211.2 |
| | | | *Ophiothrix panchyendyta** | | - | ○ | eo-14 |
| | | | *Ophiothrix trilineata* | | - | ○ | AH013212.2 |
| | | | *Ophiothrix trindadensis* | | - | ○ | MH281579.1 |
| | | | *Ophiothrix fragilis* | | - | ○ | AJ002790.1 |
| | | | *Ophiothrix quinquemaculata* | | - | ○ | AJ002795.1 |
| | | | *Ophionereis porrecta* | | - | ○ | KC760120.1 |
| | | | *Ophionereis reticulata* | | - | ○ | DQ297108.1 |
| | | | *Ophiolepis cincta* | | - | ○ | KC760088.1 |
| | | Amphiuridae | *Amphipholis squamata* | ○ | AY652510.1, FN562578.1 | | - |
| | | | *Amphiura digitula* | ○ | MH791160.1 | | - |
| | | Hemieuryalidae | *Astrogymnotes irimurai* | | - | ○ | AB605123.1 |
| | | | *Ophioplocus japonicus** | | - | ○ | eo-15 |
| | Ophiacanthida | Ophiacanthidae | *Ophiacantha antarctica* | | - | ○ | KF713455.1 |
| | | | *Ophiacantha levispina** | | - | ○ | eo-17 |
| | | | *Ophiacantha linea* | ○ | KC990833.1 | | - |
| | | | *Ophiolimna antarctica* | | - | ○ | KF713452.1 |
| | | | *Ophioplinthaca abyssalis* | | - | ○ | HM587813.1 |
| | | | *Ophioplinthaca chelys* | | - | ○ | HM587802.1 |
| | | | *Ophioplinthaca rudis** | | - | ○ | eo-22 |
| | | Ophiocomidae | *Ophiocoma brevipes* | | - | ○ | KF662926.1 |
| | | | *Ophiocoma dentata* | | - | ○ | KF662929.1 |
| | | | *Ophiocoma doederleini* | | - | ○ | KF662938.1 |
| | | | *Ophiocoma erinaceus* | | - | ○ | KF662942.1 |
| | | | *Ophiocoma krohi* | | - | ○ | KF662932.1 |
| | | | *Ophiocoma scolopendrina* | | - | ○ | KF662941.1 |
| | | | *Ophiocomella ophiactoides* | | - | ○ | KM234227.1 |
| | | | *Ophiomastix mixta* | ○ | MK343092 | | - |
| | | Ophiodermatidae | *Bathypectinura heros** | | - | ○ | eo-24 |
| | | | *Ophiarachnella gorgonia* | ○ | KC760132.1 | ○ | KC760132.1 |
| | | | *Ophioderma brevispinum* | | - | ○ | DQ297103.1 |
| | | | *Ophiopsammus maculata* | ○ | DQ297106.1 | ○ | DQ297106.1 |
| | | Ophiomyxidae | *Ophiarachna robillardi** | | - | ○ | eo-23 |
| | | | *Ophiomyxa anisacantha* | ○ | AB605124.1 | ○ | AB605124.1 |
| | | | *Ophiomyxa flaccida* | | - | ○ | DQ297104.1 |
| | | Ophiopezidae | *Ophiopeza fallax* | | - | ○ | KC760109.1 |
| | Ophioleucida | Ophioleucidae | *Ophioleuce seminudum** | | - | ○ | eo-01 |
| | Ophioscolecida | Ophiohelidae | *Ophiotholia spathifer** | | - | ○ | eo-18 |
| | | Ophioscolecidae | *Ophiologimus hexactis** | | - | ○ | eo-19 |

this setting, sites with gap were completely deleted from analysis. Finally, the new primer sets were designed to amplify the adjacent conserved region (16SOph1) and hyper-variable region (16SOph2) of 16S rRNA, using MEGA7 for 16SOph1 and Primer3web (Untergasser et al. 2012) for 16SOph2 with visual observation. 16SOph1 is located 5′ of 16SOph2, and 16SOph1-R partially overlaps with 16SOph2-F (Table 2; Suppl. material 4: fig. S1).

## Water sampling from the tanks

In order to test the detection power of the 2 newly designed primer sets (16SOph1, 16SOph2), a total of 1L of rearing water was obtained and mixed from individual aquarium tanks in MMBS in which 15 species of brittle stars were reared. The water was sampled from a total of 9 tanks. Eight of these tanks have a volume of ca. 2.5 L

**Table 2.** Nucleotide sequences of the universal primers (16SOph1 and 16SOph2). This forward (F) and reversal (R) primer pair amplifies the down-stream region of the mitochondrial 16S rRNA gene with a mean length of 112 bp (111–115 bp on average for 16SOph1) and 185 bp (180–195 bp on average for 16SOph2).

| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 16SOph1 | F | 5' | G | T | A | C | T | C | T | G | A | C | Y | G | T | G | C | A | A | A | G | G | T | A | G | C | 3' |
| | R | 5' | T | A | G | G | G | A | C | A | A | C | A | C | G | T | C | C | C | R | C | T | 3' | | | | |
| 16SOph2 | F | 5' | G | G | A | C | G | A | G | A | A | G | A | C | C | C | Y | R | T | W | G | A | G | 3' | | | |
| | R | 5' | C | A | A | C | A | T | C | G | A | G | G | T | C | G | C | A | A | A | C | 3' | | | | | |

and only one larger tank has a volume of 170 L. 1L of water was collected from each of the smaller tanks (100 mL each) and from the larger tank (200 mL); as a replicate, 50 ml of water was collected twice for each 100 ml collection and 100 ml of water was collected twice for the 200 ml collection by using 300 ml measuring cups. All water samples were taken from the surface area of the tanks. Of the 8 small tanks, 1 tank contained 2 species and 2 tanks contained 3 species of brittle stars. *Ophiomaza cacaotica*, *Astrocladus coniferus* and *Ophiothela danae* were attached to *Oxycomanthus japonicus* (sea feather), unidentified sponges and unidentified octocorals, respectively. The population of each brittle star was 1 to 10 individuals or greater. As for *Ophiothela danae*, there were definitely more than 10 individuals, but the number was too large to determine the exact number of individuals from external observation (Suppl. material 5: table S1).

## Water sampling from environmental water

In the marine field, environmental water samples from the shallow waters of Sagami Bay facing MMBS were collected from as diverse bottom substrata as possible. In this study, we collected 1L of seawater on 7 April 2021 from each of: 1) the water surface of Moroiso (35.1558°N, 139.6050°E); and 2) its rocky bottom, ca. 2 m in depth; and 3) the muddy bottom under the pier of the MMBS (35.1576°N, 139.6121°E, ca. 2 m depth). Additionally, we also collected 5L of seawater from each of: 4) the sandy and muddy bottom depths of 84.5 m (35.1552°N, 139.5766°E) on 24 September 2021; and 5) 250 m (35.1164°N, 139.5706°E) and 270 m (35.1164°N, 139.5701°E) on 8 July 2021, respectively, using the research vessel (R/V Rinkai-Maru) of MMBS with a Niskin water sampling bottle of 5L volume (K Engineering Co. Ltd.) (Figs 1, 2). The reason we sampled 5L at these depths was because we expected that deep water would be cleaner and contain less DNA than shallow water (e.g. McClenaghan et al. 2020). These are areas where the fauna of brittle stars has been well studied (e.g. Matsumoto 1917; Irimura 1982; Fujita et al. 2006).

Environmental water at the pier was collected using 12 L buckets, and when sampling under the pier with the buckets, a 10 m rope was fastened to a bucket and 500 mL of bottom water was collected twice to gather 1L of seawater (Fig. 2A). Sampling at the rocky bottom at a depth of 2 m and at the water surface at Moroiso was performed by scuba diving, and environmental water was collected underwater using 1L polychlorinated bottles (Fig. 2B, C). This "1L" volume follows a previous study (Komai et al. 2019) that did similar research on the Japanese coast. These buckets, polychlorinated bottles, and measuring cups were decontaminated thoroughly with sodium hypochlorite solution (final concentration approximately 1%) before use. The 5L Niskin water sampler bottle was decontaminated with fresh water before use (Fig. 2D). After sampling, each water sample was stirred well in the field with benzalkonium chloride solution (final concentration 0.01%) to suppress the degradation of eDNA (Yamanaka et al. 2017) and sent refrigerated to the laboratory at Environmental Research & Solution Co. Ltd. on the same day, for subsequent analysis. For the water sampling at 80 m, 250 m and 270 m, 5L of Milli-Q water was brought on the boat from the laboratory in a clean bottle as a blank sample, and the Milli-Q water was transferred to the sampling bag at the field. The blank samples were treated in the same way as field samples in subsequent analyses.

## Library preparation and MiSeq sequencing with eDNA sample

The water sample was filtered through a glass fiber filter, Whatman GF/F filters (GE Healthcare, Japan, average pore size 0.7 um). DNA extraction and purification were performed using the DNeasy and Tissue kit (Qiagen) following the protocol of Tsuji et al. (2020). After extraction, DNA was stored at -25 °C. eDNA extracted from all the environmental seawaters was subjected to the first-round PCR (1st PCR) and the second-round PCR (2nd PCR) in order to append amplified sequences with 3 kinds of adaptor sequences: 1) primer-binding sites for sequencing, 2) dual-index sequences to distinguish amplicons, and 3) sequences for binding to the flowcells of the Illumina MiSeq (Illumina, CA, USA). Before performing the 1st PCR, the DNA extract was repurified using magnetic beads using the protocol of the manufacturer of Ampure XP. The 1st PCR was carried out with a 12 µl reaction volume containing 6.0 µl KAPA HiFi HotStart ReadyMix (KAPA Biosystems, MA, USA), 1.4 µl of each 16SOph primer (5 µM primer F/R), 1.2 µl sterile Milli-Q water and 2.0 µl eDNA template. The thermal cycle profile after an initial 3 min denaturation at 95 °C was as follows: 40 cycles of denaturation at 98 °C for 20 s, annealing at 60 °C (for 16SOph1)/56 °C (for 16SOph2) for 15 s and extension at 72 °C for 15 s with the final extension at the same temperature for 5 min. The 1st PCR products were purified using Magnetic Beads (AMPure XP, Beckman) in order to remove dimers and monomers following the manu-

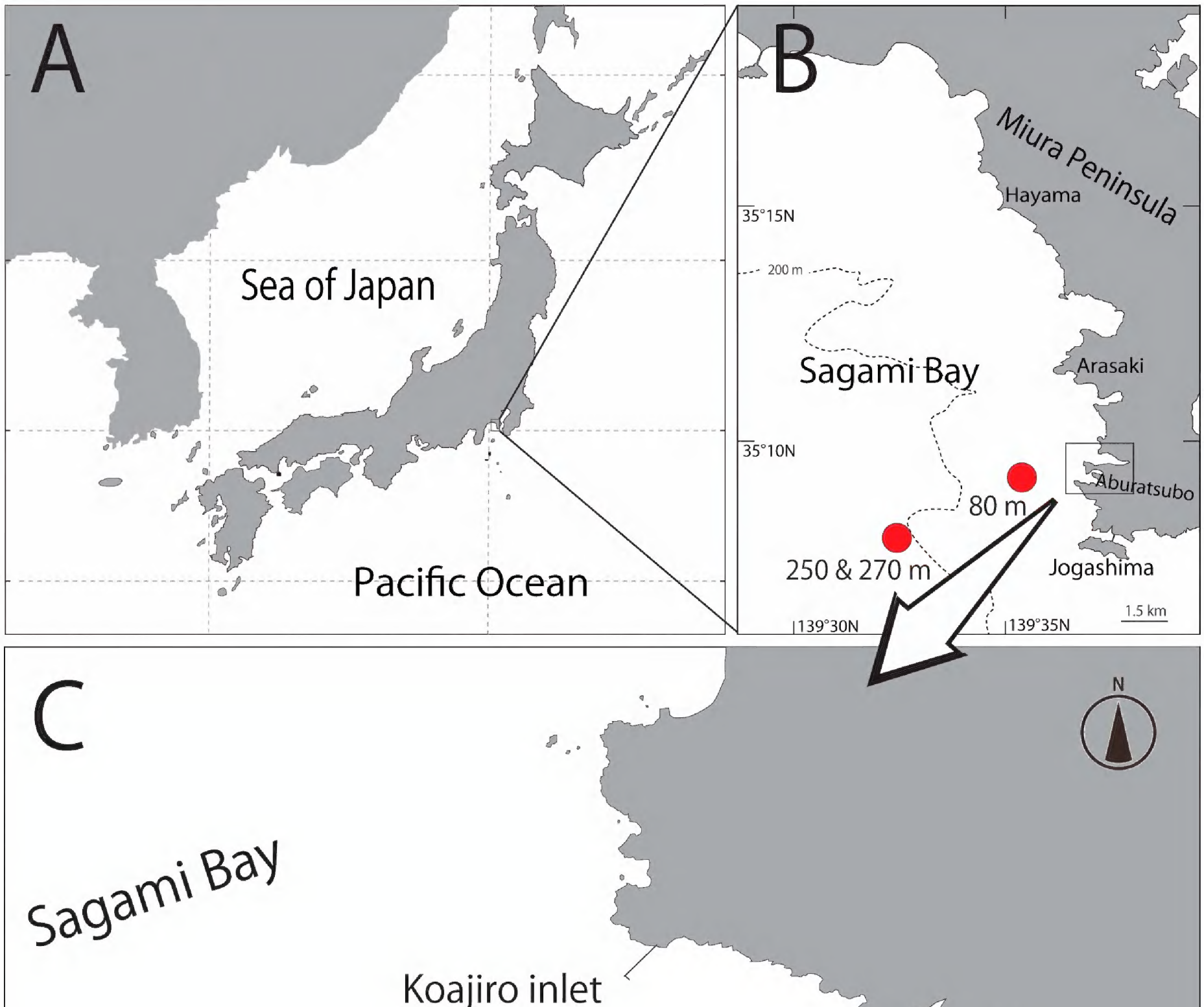


**Figure 1. A–C.** Map of Japan (**A**), showing the location of the 2 sea water sampling sites of Misaki Marina Biological Station (**B**) and 2 sea water sampling sites at shallow waters and aquarium tank (**C**) (Koajiro, Misaki, Miura, Kanagawa Prefecture).

facturer's protocol. Subsequently, the purified products were quantified using QuantiFluor ONE dsDNA System (Promega), and diluted to 0.1 ng/µl using Milli-Q water, and the diluted products were used as a template for the 2nd PCR. The 2nd PCR was conducted with a 12 µl reaction volume containing 6.0 µl KAPA HiFi HotStart ReadyMix, 0.83 µl each primer (5 µM), 2.34 µl sterile distilled $H_2O$ and 2.0 µl template. The thermal cycle profile after an initial 3 min denaturation at 95 °C was as follows: 12 cycles of denaturation at 98 °C for 20 s, annealing and extension

**Figure 2. A–D.** Natural sea water sampling operations at the Pier with a bucket, ca. 3 m (**A**); at water surface (**B**) and under water (**C**) of Moroiso with a 1 L polychlorinated bottle, ca. 2 m; at water bottom of Jogashima, ca. 80 m, 250 m and 270 m with 5L water sampling bottle (**D**). **E, F.** Aquarium tanks at Misaki Marine Biological Station, whole view (**E**) and an individual tank (**F**).

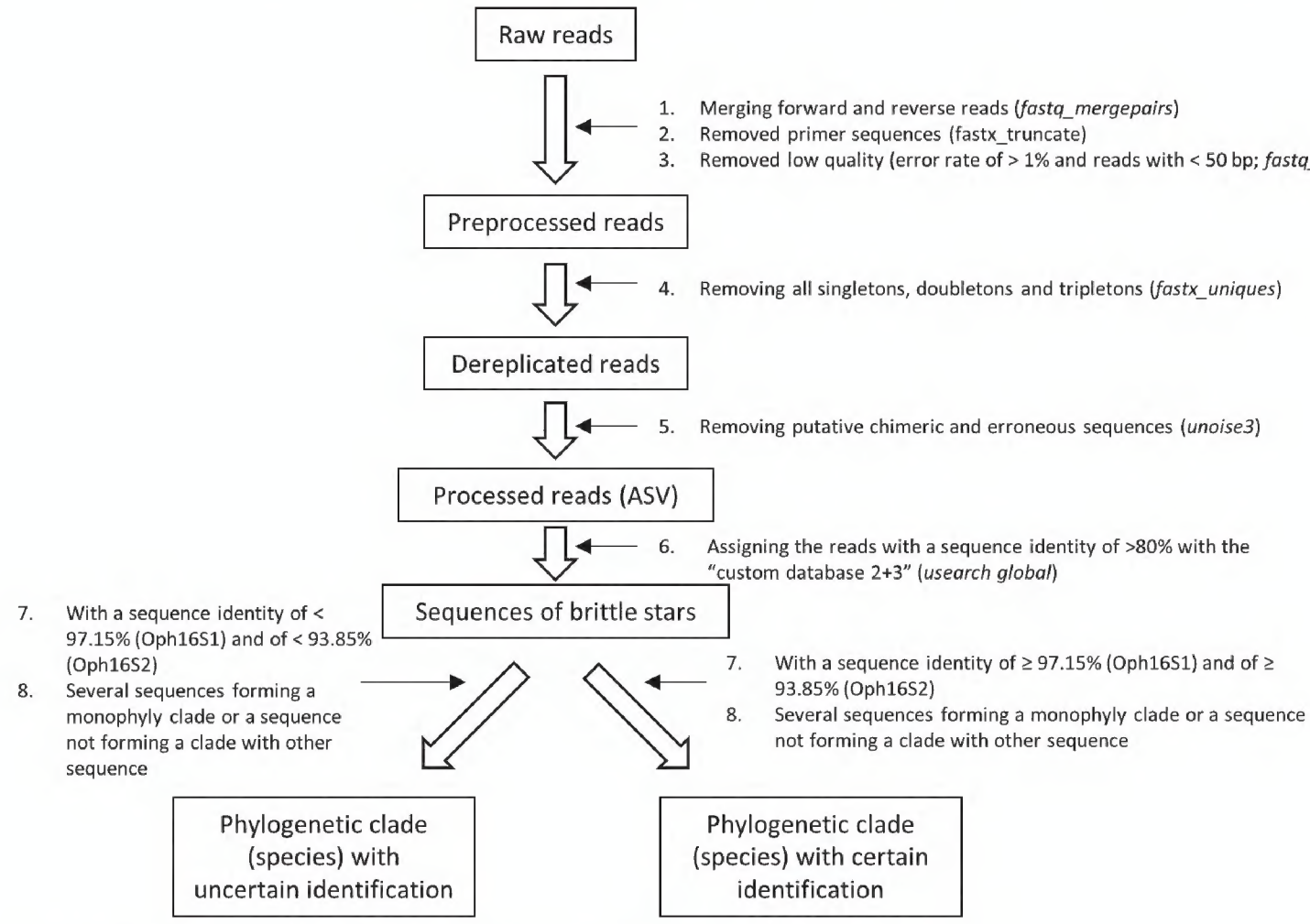
Raw reads
1. Merging forward and reverse reads (*fastq_mergepairs*)
2. Removed primer sequences (fastx_truncate)
3. Removed low quality (error rate of > 1% and reads with < 50 bp; *fastq_filter*)
Preprocessed reads
4. Removing all singletons, doubletons and tripletons (*fastx_uniques*)
Dereplicated reads
5. Removing putative chimeric and erroneous sequences (*unoise3*)
Processed reads (ASV)
6. Assigning the reads with a sequence identity of >80% with the "custom database 2+3" (*usearch global*)
Sequences of brittle stars
7. With a sequence identity of < 97.15% (Oph16S1) and of < 93.85% (Oph16S2)
8. Several sequences forming a monophyly clade or a sequence not forming a clade with other sequence
7. With a sequence identity of ≥ 97.15% (Oph16S1) and of ≥ 93.85% (Oph16S2)
8. Several sequences forming a monophyly clade or a sequence not forming a clade with other sequence
Phylogenetic clade (species) with uncertain identification
Phylogenetic clade (species) with certain identification

**Figure 3.** Graphical summary of the dataset construction protocol used in this study.

combined at 72 °C for 15 s, with the final extension at the same temperature for 5 min. In order to monitor contamination during the process of PCRs, blank samples were prepared. During the 1st PCR, a filtration blank (FB), an extraction blank (EB) and a PCR blank (1B) with 2.0 µl Milli-Q water instead of template eDNA were added. All the libraries containing the target region and the 3 adapter sequences were mixed in equal volume and the pooled libraries were size-selected from approximately 230 bp for 16SOph1 and 320 bp for 16SOph2 (including variable region+primer annealing site+NNNNNN+2nd primer binding site+slow cell binding site) using a 2% E-Gel Size Select agarose gel (Invitrogen, CA, USA). The distribution of the products of the size-selected libraries was measured and confirmed using a BioAnalyzer (Agilent Technologies) and the concentration of the size-selected libraries was measured using a QuantFlour ONE dsDNA System, and they were sequenced on the MiSeq platform using a MiSeq Reagent v2 Kit for 2×150bp PE or 2×250 PE (Illumina, CA, USA) following the manufacturer's protocol.

## Decontamination in laboratory experiment

Equipment for filtration was immersed in 5% sodium hypochlorite solution, washed with tap water, and further washed with Milli-Q water. Gamma-sterilized filter microtips were used for DNA extraction, and the DNA purification process was left to an automated extraction system (QIA cube, QIAGEN) to eliminate human error as much as possible. 1st PCR to amplify the DNA of the target taxa and the post-PCR processing were performed in separate rooms. To avoid as much as possible "Tag jumps", in which the wrong combination of tags is used in sequencing by next generation sequencing (Schnell et al. 2015), forward and reverse indexed primers (DualIndex) were used to identify samples pooled after 2nd PCR. Each pair of F primer and R primer was designed to differ by at least two bases.

## Data preprocessing and taxonomic assignment

All data preprocessing and analysis of MiSeq raw reads were performed using USEARCH v10.0.240 (Edgar 2010) according to the following steps. 1) Both forward and reverse reads were merged by aligning them using the fastq_mergepairs command. During this process, low-quality tail reads with a cut-off threshold set at a quality (Phred) score of 2, too short reads (< 50 bp) after tail trimming and those paired reads with too many differences (> 5 positions) in the aligned region (ca. 130 bp for sequences from aquarium tank by 16SOph1 and ca. 160 bp and ca. 210 bp for other sequences by 16SOph1 and 16SOph2, respectively) were discarded; 2) primer sequences were removed from those merged reads using the fastx_truncate command; 3) those reads without the primer sequences underwent quality filtering using the fastq_filter command to remove low quality reads with an expected error rate of > 1% and too short reads of < 50 bp; 4) the preprocessed reads were dereplicated using the fastx_uniques command and all singletons, doubletons and tripletons were removed from the subsequent analysis following the recommendation of Edgar (2010); 5) the dereplicated reads were denoised using the unoise3 command and all putatively chimeric and erroneous sequences were separated from the subsequent amplicon sequence variant (ASV) assignment; 6) finally, all processed reads with a sequence identity of > 80% with our "custom database 2+3" were assigned to sequences of brittle stars (given the registered name of the database) using the usearch_global command (Fig. 3; see below for the explanation of taxonomic assignment).

As reference sequences for taxonomic assignment, we used a custom database including a total of 8,975,113 mitochondrial 16S rRNA gene sequences (https://doi.org/10.6084/m9.figshare.22139909.v1; "custom database 1") from Metazoa, Plant, Fungi, Eubacteria and Archaea and 1,340 sequences from 33 known brittle star families (Suppl. material 1; "custom database 2"), which were downloaded from INSD on 17 December 2021. Therefore, custom database 1 includes the sequences of custom database 2. In addition to those published sequences, we independently determined 16SOph1 sequences from 56 species and 16SOph2 sequences from 44 species (Table 4) and added those sequences to the reference database (68 reference sequences in total; morphologically identified to 59 species from 33 genera of 16 families of 4 orders; "custom database 3"). These sequences were newly obtained by Sanger sequencing in this study (see also "Determination of target sequences of specimens collected from the study area" below for detailed methods).

A phylogenetic tree was then reconstructed using the "sequences of brittle stars" from each study area to confirm its monophyly. All sequence sets of brittle stars obtained in the data processing from each water sample were aligned using the Clustal W algorithm in MEGA7. All missing bases were scored as gaps and the sites with gaps were completely excluded from phylogenetic analysis. The substitution models were computed for each sequence set with the "find best-fit model of nucleotide substitution" option by MEGA7. Maximum likelihood analysis (ML) with 1000 bootstrap replicates was performed with MEGA7 to reconstruct the ML phylogenetic tree. The trees were visualized with MEGA 7. Monophyly of each node in the phylogenetic trees was considered to be supported if bootstrap was higher than 80%. Node bootstrap values lower than 79% were considered as not monophyletic (Suppl. material 4: figs S2–S10).

To determine the species "threshold distance" we measured the genetic distance for each 16SOph1 and 16SOph2 region of custom database 3 to obtain the minimum genetic distance interspecies. We refrain from using custom database 2 since it was expected to include far fewer sequences from Sagami Bay, unidentified sequences such as "Ophiuroidea sp.", and misidentified sequences. If a genetic distance between a sequence from custom database 3 and a sequence from the eDNA was estimated to be within the "threshold distance", the latter sequence was given the taxon name in the database, and treated as "species with certain identification". Taxa including all biota other than the brittle stars were searched with the 2 primer sets as sequences that matched our custom database 1 with more than 97% similarity.

### Determination of target sequences of specimens collected from the study area

The DNA sequences of the 16S rRNA gene were determined for 59 morphologically identified species that had been collected in Sagami Bay and from similar environments in the seas around Japan during the last 10 years. This included specimens from areas where water samples were collected (including tanks) for the present study. The method of DNA extraction followed that of Okanishi and Fujita (2013). The primer set 16Sar (5′-CGC CTG TTT ATC AAA AAC AT-3′) and 16Sbr (5′-CCG GTC TGA ACT CAG ATC ACG T-3′) (approximately 500 bp on the 5′ side of the 16S rRNA gene) was used and its amplicon region covered the target regions of both 16SOph1 and 16SOph2 (Palumbi 1996). For specimens that 16Sar and 16Sbr would not amplify, an alternative newly designed internal primer (Oph-16S-R1) covering 16SOph1 and 16SOph2 was used. The primer sets used for amplifying the sequence including 16SOph1 and/or 16SOph2 are summarized in Table 3 and Suppl. material 4: fig. S1. The optimum cycling parameters for those primer sets consisted of an initial denaturation step of 95 °C/2 min followed by 41 cycles of 95 °C/30 s, 48 °C/40 s and 72 °C/1 m with final extension step at 72 °C/10 m, which was followed by storage at 10 °C (Okanishi and Fujita 2013). The PCR products were separated from excess primers and oligonucleotides using Exo-SAP-IT (GE Healthcare), following the manufacturer's protocol. Sequences amplified with 16Sar and 16Sbr were sequenced bidirectionally and sequence products were run on a 3730xI DNA Analyzer (Thermo Fisher Scientific). The accession numbers of DNA Data Bank of Japan (DDBJ) of the examined specimens were deposited in INSD with collection locality data (Table 4). For all examined materials, coordinates, depth, date, collector, gear, and identifier were provided in as much detail as possible (Suppl. material 3).

## Results

### Development of 16SOph primers

As a result of aligning the mitochondrial 16S rRNA sequences from datasets of 132 species of brittle stars (Table 1), we were able to design new primer sets for the conserved region of about 110 bp and the hyper-variable region of about 180 bp. These primer sets were named "16SOph1" and "16SOph2", respectively (Table 3). Melting temperatures and G/C contents of 16SOph1-F/16SOph1-R/16SOph2-F/16SOph2-R were

**Table 3.** Primers and PCR conditions for amplifying the two target regions in this study, 16SOph1 and 16SOph2.

| Forward primer | | Reverse primer | | Annealing temperature (°C) | Coverage | |
|---|---|---|---|---|---|---|
| Name | Sequence | Name | Sequence | | 16SOph1 | 16SOph2 |
| 16Sar | 5‘-CGCCTGTTTACCAAAAACAT-3‘ | 16Sbr | 5‘-CCGGTCTGAACTCAGATCACGT-3‘ | 46 | ✓ | ✓ |
| 16SOph1-F | 5‘-GTACTCTGACYGTGCAAAGGTAGC-3‘ | Oph-16S-R1 | 5‘-TGATCCAACATMGAGGTCGCAA-3‘ | 46 | ✓ | ✓ |
| 16Sar | 5‘-CGCCTGTTTACCAAAAACAT-3‘ | 16SOph1-R | 5‘-TAGGGACAACACGTCCCRCT-3‘ | 46 | ✓ | |

**Table 4.** A list of 68 species, including 59 MMBS brittle star species collected from Sagami Bay in the last 10 years, with 4 species („*“) from similar environment around Japan and 3 species („⁂“) with high identities with environmental DNA sequences. Based on the MMBS specimen, occurance records of the examined area are marked with an „○“ . For each species, „✓“ is provided when the partial 16S rRNA sequence containing 16SOph1, 16SOph2, or both has been sequenced. „⁂“indicates the detection of multiple cryptic species; „⁂“ indicates species number including cryptic species.

| Superorder | Order | Family | Species | eDNA sampling area | | | | | | | | | | | | | | | | Specimen no | Depth range (m) | | 16S sequence | | | Accession number | Sampling locality other than Sagami Bay |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Tank | 1 | 2 | Moroiso sur. | 1 | 2 | Moroiso bottom | 1 | 2 | Pier | 1 | 2 | 80 m | 1 | 2 | 250–270 m | 1 | | | 16SOph1 | 16SOph2 | 16SOph1-2 | | |
| Euryophiurida | Euryalida | Gorgonocephalidae | *Astrocladus dofleini* | ○ | 1 | 2 | ○ | | | ○ | | | | | | ○ | | | | | NSMT E-5480 | 4 | 80 | | | | AB605105 | Wakayama |
| | | | *Astrodendrum spinulosum*[1] | | | | | | | | | | | | | | 1 | | | 1 | NSMT E-6273 | ca 60 | ca 60 | | | | AB605110 | Otsuchi (Japan) |
| | | Euryalidae | *Astroceras annulatum*[1] | | | | | | | | | | | | | | 1 | | | | NSMT E-6261 | 175 | 176 | | | | AB605089 | Ogasawara (Japan) |
| | | Ophiosphalmidae | *Ophiosphalma cancellatum* | | | | | | | | | | | | | ○ | | | ○ | | NSMT E-13937 | 60 | 473 | ✓ | ✓ | ✓ | LC749645 | |
| | | Ophiuridae | *Ophiura calyptolepis* | | | | | | | | | | | | | | | | ○ | 1 | NSMT E-13938 | 147 | 360 | ✓ | | | LC749621 | |
| | | | *Ophiura cryptolepis* | | | | | | | | | | | | | | | | | | NSMT E-13939 | 473 | 818 | ✓ | ✓ | | LC749622, LC749671 | |
| | | | *Ophiura ooplax* | | | | | | | | | | | | | | | | | | NSMT E-13940 | 340 | 604 | ✓ | | | LC749623 | |
| | | | *Ophiura paucisquama* | | | | | | | | | | | | | | | | | | NSMT E-13941 | nd | nd | ✓ | | | LC749611 | |
| | | | *Ophiuroglypha kinbergi* | | | | | | 2 | | 1 | 2 | | | | ○ | 1 | 2 | | 1 | NSMT E-13942 | 4 | 89.1 | ✓ | ✓ | ✓ | LC749659 | |
| | Ophiurida | Ophiopyrgidae | *Amphiophiura penichra* | | | | | | | | | | | | | | | | | | NSMT E-13943 | 440 | 541 | ✓ | | | LC749624 | |
| | | | *Ophiomusium scalare* | | | | | | | | | | | | | | | | | | NSMT E-13944 | 106 | 198 | ✓ | ✓ | ✓ | LC749638 | |
| | | | *Stegophiura sladeni* | | | | | | | | | | | | | ○ | 1 | | ○ | | NSMT E-13945 | 79 | 396 | ✓ | ✓ | ✓ | LC749630 | |
| | | | *Stegophiura sterea* | | | 2 | | | | | | | | | | | 1 | | ○ | 1 | NSMT E-13946 | 251 | 541 | ✓ | ✓ | ✓ | LC749640 | |
| | | | *Stegophiura vivipara* | | | | | | | | | | | | | ○ | | | ○ | | NSMT E-13947 | 46.8 | 320 | ✓ | ✓ | ✓ | LC749646 | |
| | | | *Ophiosparte gigas*⁂ | | | | | | | | | | | | | | | | | 1 | nd | nd | nd | ✓ | ✓ | ✓ | GU226990 | Ross sea |
| | Ophintegrida | Ophiotrichidae | *Macrophiothrix longipeda* | ○ | 1 | 2 | ○ | 1 | 2 | ○ | 1 | 2 | ○ | | 2 | | 1 | 2 | | | NSMT E-13948, NSMT E-13949 | 3 | 3 | ✓ | ✓ | ✓ | LC749647, LC749634, AY365160 | |
| | | | *Ophiothela danae* | ○ | 1 | 2 | ○ | | 2 | ○ | 1 | 2 | | 1 | 2 | | | | | | NSMT E-14164, MO-2016-8 | 2 | 25 | ✓ | ✓ | ✓ | LC749633, LC756974 (Shirahama, Japan) | |
| | | | *Ophiothrix panchyendyta*1, 2 | ○ | 1⁂ | 2 | | 1 | 2 | | 1 | 2 | | 1 | 2 | ○ | 1 | | ○ | | NSMT E-13950, NSMT E-13951 | 86.6 | 365 | ✓ | ✓ | ✓ | LC749648, LC749637 | |
| | | | *Ophiothrix exigua* | ○ | 1 | 2 | ○ | 1 | | ○ | | 2 | | | | ○ | | | | | NSMT E-13952 | 0 | 88 | ✓ | ✓ | ✓ | LC749663 | |
| | | | *Ophiothrix nereidina* | ○ | 1 | 2 | ○ | 1 | 2 | ○ | 1 | 2 | | 1 | 2 | | | 2 | | 1 | NSMT E-13953 | 4 | 11 | ✓ | ✓ | ✓ | LC749653 | |
| | | | *Ophiomaza cacaotica* | ○ | 1 | 2 | | | | | 1 | | | | | | | | | | MO-2021-2 | 10 | 10 | ✓ | ✓ | ✓ | LC749664 | |
| | | Amphilepididae | *Amphipholis squamata* | ○ | | | ○ | 1 | 2 | ○ | 1 | 2 | ○ | 1 | 2 | ○ | | | ○ | 1 | MO-2021-15 | 0 | 484 | | | | AY652510, FN562578 | |
| | | | *Amphipholis kochii* | | | | | | | | | | | | | ○ | 1 | | ○ | | NSMT E-13954 | 2 | 755 | ✓ | ✓ | ✓ | LC749650 | Southwestern Japan |
| | | | *Amphioplus japonicus* | | | | ○ | | | ○ | | | | | | ○ | 1 | | | | NSMT E-13955 | 0.8 | 91.4 | ✓ | | | LC749619 | |
| | | | *Amphioplus macraspis* | | | | | | | | | | | | | | | | | | NSMT E-13956 | 314 | 509 | ✓ | ✓ | | LC749615, LC749670 | |
| | | | *Amphioplus rhadinobrachius* | | | | | | | | | | | | | | | | ○ | | NSMT E-13957 | 230 | 398 | ✓ | ✓ | | LC749613, LC749672 | |
| | | Amphiuridae | *Amphichilus trichoides*[1] | | | | | | | | | | | | 2 | | 1 | 2 | | | NSMT E-13958 | 71 | 75.5 | ✓ | ✓ | ✓ | LC749627 | Shirahama (Japan) |
| | | | *Amphiura ancistrotus* | | | | | | | | | | | | | ○ | 1 | | ○ | | NSMT E-13959 | 85 | 756 | ✓ | ✓ | ✓ | LC749642 | |
| | | | *Amphiura archystata* | | | | | | | | | | | | | | | | ○ | | NSMT E-13960 | 255 | 510 | ✓ | ✓ | ✓ | LC749655 | |
| | | | *Amphiura bellis* | | | | | | | | | | | | | | | | ○ | | NSMT E-13961 | 221 | 541 | ✓ | ✓ | | LC749610, LC749673 | |
| | | | *Amphiura carchara* | | | | | | | | | | | | | | | | | | NSMT E-13962 | 316 | 604 | | ✓ | | LC749643 | |
| | | | *Amphiura euopla* | | | | | | | | | | | | | ○ | | | ○ | | NSMT E-13963 | 4.1 | 328 | ✓ | ✓ | ✓ | LC749658 | |

| Superorder | Order | Family | Species | eDNA sampling area | | | | | | | | | | | | | | | | | Specimen no | Depth range (m) | | 16S sequence | | | Accession number | Sampling locality other than Sagami Bay |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Tank | 1 | 2 | Moroiso sur. | 1 | 2 | Moroiso bottom | 1 | 2 | Pier | 1 | 2 | 80 m | 1 | 2 | 250–270 m | 1 | | | | 16SOph1 | 16SOph2 | 16SOph1-2 | | |
| Ophintegrida | Amphilepidida | Amphiuridae | *Amphiura digitula*⁂ | | | | | | | | | | | | | | 1 | 2 | | | nd | nd | nd | | | | MK343096 | South Korea |
| | | | *Amphiura iridoides* | | | | | | | | | | | | | ○ | | | ○ | | NSMT E-13964 | 94.7 | 541 | ✓ | | | LC749620 | |
| | | | *Amphiura koreae* | | | | | | | | | | | | | | | | ○ | | NSMT E-13965 | 93.6 | 770 | ✓ | ✓ | ✓ | LC749651 | |
| | | | *Amphiura sinicola*⁂ | | | | | **1** | | | | | | | | | | | | | nd | nd | nd | | | | MK343094 | South Korea |
| | | | *Amphiura trachydisca* | | | | | | | | | | | | | | | | ○ | 1 | NSMT E-13966 | 80 | 320 | ✓ | ✓ | ✓ | LC749665 | |
| | | | *Amphiura vadicola* | | | | | | | | | | ○ | 1 | 2 | ○ | | | | | NSMT E-13967 | 2 | 78.3 | ✓ | ✓ | ✓ | LC749632 | |
| | | | *Amphiura* sp. | | | | | | | | | | | | | ○ | 1 | | | | NSMT E-13968 | 83.5 | 89.3 | ✓ | | | LC749614 | |
| | | | *Ophiocentrus* sp. | | | | | | | | | | | | | | 1 | 2 | | | NSMT E-13969 | nd | nd | ✓ | ✓ | ✓ | LC749661 | |
| | | | *Ophiocentrus verticillatus* | | | | | | | | | | | | | ○ | | | ○ | | NSMT E-13970 | 18.6 | 256 | ✓ | ✓ | ✓ | LC749641 | |
| | | | *Ophiocentrus tokiokai** | | | | | | 2 | | | | | | | | 1 | 2 | | | NSMT E-13971 | 10 | 30 | ✓ | ✓ | ✓ | LC749629 | Kochi (Japan) |
| | | Ophiactidae | *Ophiactis brachygenys* | | 1 | 2 | | | | | | | | | | | | | | | NSMT E-13972 | 504 | 551 | ✓ | ✓ | ✓ | LC749657 | |
| | | | *Ophiactis dyscrita* | | | | | | | | | | | | | | | | ○ | | NSMT E-13973 | 60 | 309 | | ✓ | | LC749667 | |
| | | | *Ophiactis lymani*⁂ | | | | | | 2 | | | 2 | | | 2 | | | | | | nd | nd | nd | | | | KP128040 | Brazil |
| | | | *Ophiactis macrolepidota*1, 2 | | 1 | | ○ | 1⁂ | 2 | ○ | 1⁂ | 2 | | 1 | 2 | | | | | | NSMT E-13974, NSMT E-13975 | 30 | 30 | ✓ | ✓ | ✓ | LC749625, LC749666 | |
| | | | *Ophiactis profundi* | | | 2 | | | | | | | | | | ○ | 1 | | ○ | | NSMT E-13976 | 85 | 309 | ✓ | | | LC749626 | |
| | | | *Ophiactis savignyi* | | | | ○ | 1 | 2 | ○ | 1 | 2 | ○ | 1 | 2 | | 1 | 2 | | | NSMT E-13977 | 0 | 30 | ✓ | ✓ | ✓ | LC749631 | |
| | | Ophionereididae | *Ophionereis porrecta* | | | | ○ | | | ○ | | | | | | | | | ○ | | NSMT E-13978 | 18.6 | 85.7 | ✓ | | | LC749612 | |
| | | | *Ophionereis dubia* | | | | ○ | 1 | 2 | ○ | 1 | | | | | | | | | | NSMT E-13979 | 5 | 15 | ✓ | ✓ | ✓ | LC749633 (Shirahama, Japan), LC749656 | |
| | | Ophiopholidae | *Ophiopholis aculeata* | | | | | | | | | | | | | | | | ○ | | NSMT E-13844 | 267 | 600 | ✓ | ✓ | | LC749607, LC749668, MK343095 | |
| | | | *Ophiopholis mirabilis* | | | | | | | | | | | | | ○ | | | | | NSMT E-13980 | 3 | 85.7 | ✓ | | | LC749605, MK343098 | |
| | | | *Ophiopholis bracyactis* | | | | | | | | | | | | | | | | ○ | | NSMT E-13981 | 93.6 | 365 | ✓ | | | LC749606 | |
| | | Ophiopsilidae | *Ophiopsila squamifera* | | | | | | | | | | | | | ○ | | | | | NSMT E-13982 | 80.7 | 106 | ✓ | | | LC749617 | |
| | | Hemieuryalidae | *Ophiozonella longispina* | | | | | | | | | | | | | | | | | | NSMT E-13845 | nd | nd | ✓ | | | LC749618 | |
| | | | *Ophiozonella projecta* | | | | | | | | | | | | | ○ | | | | | NSMT E-13983 | 108 | 187 | ✓ | ✓ | ✓ | LC749639 | |
| | | | *Ophioplocus japonicus* | ○ | | 2 | ○ | | 2 | ○ | | 2 | | | | | | | | | NSMT E-13984 | 0 | 3.5 | ✓ | ✓ | ✓ | LC749662 | |
| | Ophiacanthida | Ophiacanthidae | *Ophiacantha levispina* | | | | | | | | | | | | | ○ | | | ○ | | NSMT E-13985 | 86.6 | 300 | ✓ | ✓ | ✓ | LC749654 | |
| | | | *Ophiacantha rhachophora* | | | | | | | | | | | | | | | | | | NSMT E-13986 | 340 | 380 | ✓ | ✓ | | LC749609, LC749669 | |
| | | | *Ophiacantha stellefera* | | | | | | | | | | | | | | | | | 1 | NSMT E-13987 | 300 | 504 | ✓ | ✓ | ✓ | LC749649 | |
| | | | *Ophiopthalmus normani* | | | | | | | | | | | | | | | | | | NSMT E-13988 | 563 | 1009 | ✓ | | | LC749608 | |
| | | Ophiodermatidae | *Ophiarachnella gorgonia* | ○ | 1 | 2 | ○ | | | ○ | | | | | | | | | | | NSMT E-13989 | 0.3 | 20 | ✓ | ✓ | ✓ | LC749636, KC760132 | |
| | | | *Ophiopsammus anchista* | ○ | | | | | | | | | | | | ○ | | | ○ | 1 | NSMT E-13990 | 80 | 250 | ✓ | ✓ | ✓ | LC749660 | |
| | | Ophiomyxidae | *Ophiomyxa anisacantha* | | | | | | | | | | | | | | | | ○ | | NSMT E-13991 | 251 | 756 | ✓ | | | LC749616, AB605124 | |
| | | | *Ophiodera australis* | | | | | | | | | | | | | | | | | | NSMT E-13992 | 153 | 200 | ✓ | ✓ | ✓ | LC749644 | |
| | | Ophiocomidae | *Ophiomastix mixta* | ○ | 1 | 2 | ○ | 1 | 2 | ○ | 1 | 2 | | 1 | | | 1 | 2 | | | NSMT E-13993 | 0 | 3 | ✓ | ✓ | ✓ | LC749628 | |
| | | | *Ophiocoma dentata* | ○ | 1 | 2 | ○ | | | ○ | | | | | | | | | | | NSMT E-13994 | 0 | 4 | ✓ | ✓ | ✓ | LC749652 | |
| Superorder unidentified | | | Ophiuroidea sp.⁂ | | 1 | 2 | | | 2 | | | 2 | | 1 | 2 | | | 2 | | | nd | nd | nd | | | | RCMBAR668, 2231 | |
| Total | | | | 13 | 14⁂ | 15 | 15 | 11⁂ | 14 | 15 | 12⁂ | 13 | 4 | 9 | 11 | 22 | 18 | 10 | 25 | 10 | | | | | | | | |

63.8 °C/62.5 °C/59.9 °C/64.3 °C and 52%/58%/61%/53%, respectively. The length of the 16SOph sequence data obtained from environmental water was 111–115 bp (112 bp on average) for 16SOph1 and 180–195 bp (185 bp on average) for 16SOph2. These sequences were registered in DDBJ/EMBL/NCBI (Table 4).

### Threshold setting

The genetic distance of custom database 2 was examined, and 22,360 (16SOph1) and 6,510 (16SOph2) sequence combinations were calculated with distance of 0, which indicates they matched 100% in sequence. Among these, many combinations were found that differed at the order level, indicating that they were probably misidentified (Suppl. material 5: tables S2, S3). The genetic distance of the sequences in custom database 3 showed that most combinations with distance 0 were the same species. However, the genetic distance between *Amphiura koreae* and *Amphioplus rhadinobrachius* was also calculated as 0 (16SOph1; Suppl. material 5: table S4). In addition, even within the same species (*Ophiothela danae*), genetic distances of 1.87% (16SOph1) and 0.63% (16SOph2) were calculated depending on the difference of collection sites, and some degree of genetic distance was calculated between *A. trachydisca* and *A. koreae* (1.87% for 16SOph1; 1.2% for 16SOph2), and between *A. rhadinobrachius* and *A. trachydisca* (1.87% for 16SOph1) (Suppl. material 5: tables S4, S5). Therefore, in this study, these genetic distances (1.87% for 16SOph1 and 1.2% for 16SOph2) were considered to indicate intraspecific variation, and the three species of Amphiuridae (*A. koreae*, *A. trachydisca* and *A. rhadinobrachius*) were considered synonyms (see also Discussion for this taxonomic issue).

Except for these three species of Amphiuridae, the smallest genetic distances were 2.86% (16SOph1) and 6.15% (16SOph2) between *Stegophiura sladeni* and *Stegophiura sterea* (Suppl. material 5: tables S4, S5). Therefore, in this study, we set 97.14% and 93.85% as the threshold values for the species boundary of 16SOph1 and 16SOph2, respectively, and the eDNA sequences in the database with similarities exceeding this value were considered the same species.

## Taxa detection from water eDNA

### Total reads

After merged, quality-filtered, dereplicated and denoised the raw reads, we obtained, respectively for 16SOph1/16SOph2, a total of 362,300/574,409 usable processed reads for aquarium tank, 141,629/152,491 for the sea surface of Moroiso, 142,856/157,812 for the sea bottom of Moroiso, 134,055/281,242 for the Pier, and 360,480/160,237 for the 80 m depth, respectively. The number of usable processed reads obtained for 250–270 m sample was 177,844 (only 16SOph1).

### Taxa detected with 16SOph1 from aquarium tanks and environmental waters around MMBS

We compared the processed reads (hereafter "reads") obtained from eDNA of environmental waters (including aquarium water tanks) at MMBS with our custom database 2+3. As a result, we recorded the following number of sequences of brittle stars from this database that matched with >80% similarity (hereafter "sequences") the eDNA reads: a total of 20 sequences from the tank (comprising a total of 338,367 reads, the range was 167–278,769, and the average was 21,147 reads), 37 sequences from Moroiso (totals of 94,056 and 109,680 reads, the range was 162–25,618 and 62–26,844, and the average was 2,542 and 2,964 reads from surface and bottom, respectively), 19 sequences from the Pier (a total of 86,056 reads, the range was 15–30,422, and the average was 7171 reads), 52 sequences from 80 m depth (a total of 182,951 reads, the range was 4–108,554, and the average was 4,691 reads), and 20 sequences from 250 m and 270 m depths (a total of 37,211 reads, the range was 5–10,824, and the average was 1,162 reads) (Suppl. material 5: tables S6–S10). The sampling points at 250 m and 270 m depths were geographically close to each other, and we combined the data from the 2 areas.

We constructed maximum likelihood phylogenetic trees for these sequences of brittle stars in each study area and filtered the obtained monophyletic sequences by the "threshold" value, resulting in: 16 phylogenetic clades in the tank, 14 of which could be considered as species with certain identification (Suppl. material 4: fig. S2; Suppl. material 5: table S6); 18 phylogenetic clades in Moroiso, 14 of which could be considered as species with certain identification (Suppl. material 4: fig. S3; Suppl. material 5: table S7); 12 phylogenetic clades in Pier, 9 of which could be considered as species with certain identification (Suppl. material 4: fig. S4; Suppl. material 5: table S8); 39 phylogenetic clades in the 80 m sample, 18 of which could be considered as species with certain identification (Suppl. material 4: fig. S5; Suppl. material 5: table S9); 16 phylogenetic clades in the 250 and 270 m sample, 10 of which could be considered as species with certain identification (Suppl. material 4: fig. S6; Suppl. material 5: table S10). In Moroiso, for the clade of *Macrophiothrix longipeda*, the bootstrap value was 67. However, considering that the similarity range of the clade was 97.3–100, we considered this clade to be a species with certain identification. In the Pier sample, for the clade of *Amphiura iridoides* the bootstrap value was 79. However, because the difference in nucleotide sequence was within 2 bp, we consider it to be a species with certain identification. In the 80 m depth sample, the range of identity of 3 sequences of *Amphiura digitula* obtained was 93.8–100%, and this value is below the 16SOph1 threshold. However, these 3 sequences form a monophyletic clade in the phylogenetic tree, and their maximum similarity was 100%. Therefore, they were considered to be a species with certain identification (Suppl. material 5: tables S6–S10).

Among them, 10/13 species, 8/15 species, 3/9 species, 7/18 species, and 5/10 species were confirmed to be reared in the tank, or distributed in Moroiso, the Pier, 80 m, and 250–270 m, respectively (Suppl. material 5: tables S6–S10).

## Taxa detected with 16SOph2 from aquarium tanks and environmental waters around MMBS

We compared the processed reads obtained from eDNA of environmental waters (including the aquarium tanks at MMBS) with our custom database 2+3. As a result, a total of 36 sequences of brittle stars from the tank (comprising a total of 522,242 reads, the range was 18–482,876, and the average was 34,816 reads), 42 sequences from Moroiso (totals of 6,147 and 13,460 reads, the ranges were 13–2,397 and 8–4,353, and the averages were 409 and 897 reads from the surface and bottom, respectively), 14 sequences from the Pier (a total of 11,186 reads, the range was 8–4,065, and the average was 1,016 reads), and 16 sequences from 80 m (a total of 755 reads, the range was 4–575, and the average was 75 reads) (Suppl. material 5: tables S11–S13) were found to match our custom database with more than 80% similarity. Our maximum likelihood phylogenetic tree based on sequences in each study area and filtration of the obtained monophyletic sequences by the "threshold" value resulted in: 15 phylogenetic clades in the tank, 15 of which could be considered as species with certain identification (Suppl. material 4: fig. S7); 15 phylogenetic clades in Moroiso, 15 of which could be considered as species with certain identification (Suppl. material 4: fig. S8); 11 phylogenetic clades in the Pier, 11 of which could be considered as species with certain identification (Suppl. material 4: fig. S9); and 10 phylogenetic clades in the 80 m depth sample, 10 of which could be considered as species with certain identification (Suppl. material 4: fig. S10).

Among them, 11/15 species, 10/15 species, 4/11 species, and 1/10 species were confirmed to be reared in the tank, or distributed in Moroiso, at the Pier, and at 80 m depth, respectively (Suppl. material 5: tables S11–S14). In 80 m, the range of identity of 7 sequences of *Amphichilus trichoides* was 92.1–100%, and this value is below the 16sOph2 threshold. However, these 7 sequences form a monophyletic clade in the phylogenetic tree, and their maximum similarity was 100%. Therefore, they were treated as a certainly identified species.

## Other detected taxa apart from Ophiuroidea

The results of the search of all other biota other than the brittle stars for 16SOph1 indicated that almost all of the detected species were brittle stars in all environmental waters, and 1 insect species was detected at Moroiso and in the deep sea, respectively, which may have been due to contamination during the experimental analysis (Fig. 4). Two species of nemerteans were detected in the aquarium tanks. This may have been due to the species that were reared together with the brittle stars in the tank. On the other hand, only brittle stars were detected at the Pier and 80 m (Fig. 4). Non-metazoa biota (Plant, Fungi, Eubacteria, and Archaea) were not detected using 16SOph1.

The proportion of OTUs (species)/reads putatively assigned to 1) brittle star was 87.5%/99.9% (Tanks), 91.6%/99.9% (Pier), 100%/100% (Moroiso surface), 90.9%/99.9% (Moroiso bottom), 100%/100% (80 m) and 91.6%/99.9% (deep sea); 2) Echinodermata other than brittle star was 0% for all sites; 3) other metazoans was 12.5%/>0.001% (Tanks), 8.3%/>0.001% (Moroiso surface), 9%/>0.001% (Moroiso bottom) and 8.3%/>0.001% (deep sea) (Fig. 4; Table 5). A wide variety of species were detected with 16SOph2. In addition to the brittle

**Table 5.** A list of all reads and species detected with 16SOph1 and 16SOph2, compared with the custumed data including 16S sequences of all biota.

| Taxa | | 16S1 Tank | | Moroiso_ surface | | Moroiso_ bottom | | Pier | | 80 m | | Deepsea | | 16S2 Tank | | Moroiso_ surface | | Moroiso_ bottom | | Pier | | 80 m | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Phylum | Class | reads | spp. | reads | spp. | reads | spp. | reads | spp. | reads | spp. | reads | spp. | reads | spp. | reads | spp. | reads | spp. | reads | spp. | reads | spp. |
| Echinodermata | Ophiuroidea | 338367 | 14 | 109680 | 11 | 94056 | 10 | 86056 | 9 | 182951 | 18 | 37211 | 11 | 522242 | 15 | 5791 | 14 | 11116 | 13 | 11186 | 11 | 755 | 10 |
| Echinodermata | Echinoidea | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 46 | 1 | 17 | 2 | 137 | 5 | 13 | 1 | 0 | 0 |
| Echinodermata | Holothuroidea | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1835 | 3 | 52 | 2 | 451 | 2 | 752 | 2 | 0 | 0 |
| Echinodermata | Asteroidea | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 | 1 | 5 | 1 | 0 | 0 |
| Nemertea | Pilidiophora | 118 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Nemertea | Paleonemertea | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 12 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Arthropoda | Insecta | 0 | 0 | 7 | 1 | 60 | 1 | 0 | 0 | 0 | 0 | 9 | 1 | 111 | 1 | 15985 | 1 | 3798 | 1 | 288 | 1 | 0 | 0 |
| Arthropoda | Crustacea | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 79 | 2 | 364 | 9 | 707 | 6 | 4203 | 2 | 13 | 1 |
| Bryozoa | Gymnolaemata | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 117 | 1 | 12133 | 5 | 1514 | 5 | 16966 | 7 | 0 | 0 |
| Chordata | Actinopterygii | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 1 | 0 | 0 | 4 | 1 | 82 | 8 | 0 | 0 |
| Chordata | Mammalia | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 17 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cnidaria | Anthozoa | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 14 | 1 | 0 | 0 | 5 | 1 | 0 | 0 | 0 | 0 |
| Mollusca | Cephalopoda | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 23 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mollusca | Gastropoda | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 23 | 5 | 510 | 17 | 8533 | 13 | 1187 | 12 | 0 | 0 |
| Mollusca | Bivalvia | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 58 | 1 | 0 | 0 | 19585 | 8 | 0 | 0 |
| Porifera | Demospongia | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 416 | 3 | 863 | 5 | 964 | 6 | 1193 | 4 | 0 | 0 |
| Annelida | Polychaeta | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8 | 1 | 12 | 2 | 0 | 0 | 0 | 0 |
| Heterokontophyta | Pyramimonadophyceae | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 35 | 3 | 85 | 2 | 95 | 1 | 0 | 0 |
| Chlorophyta | Mamiellophyceae | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Proteobacteria | undetermined | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 252 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

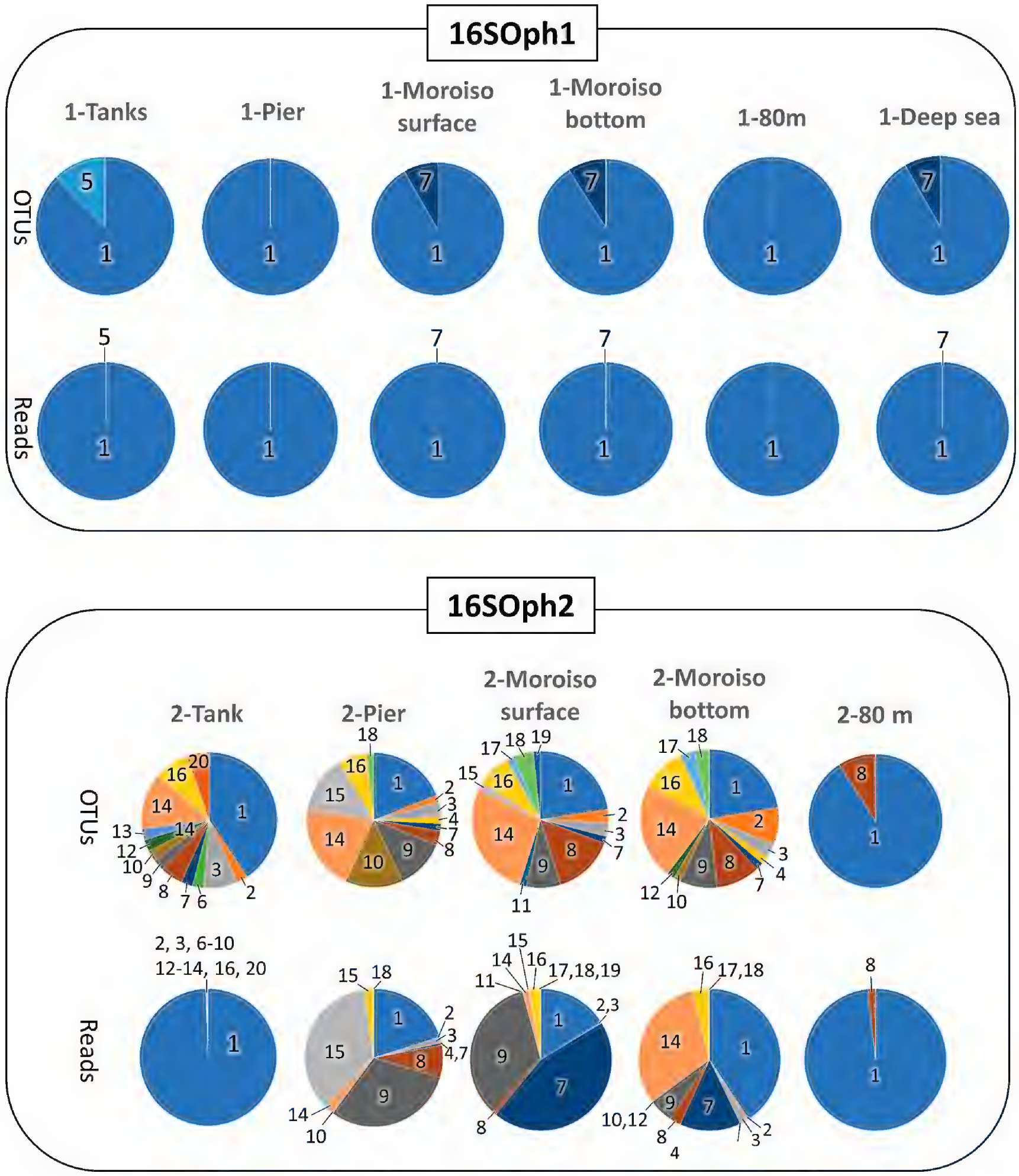


**Figure 4.** Compositions of OTUs (species) and reads of the all biota (including detected brittle star species with certain identification in this study) detected from eDNA around MMBS by 16SOph1 and 16SOph2.

stars: 20 species (8 phyla, 11 classes) were detected in the tank, 43 species (6 phyla, 9 classes) in the surface of Moroiso, 43 species (8 phyla, 11 classes) in the bottom of Moroiso, and 46 species (6 phyla, 10 classes) in the Pier. On the other hand, only 1 species of crustacean (Arthropoda) was detected at 80 m (Fig. 4). The number of brittle star species detected was more than one-quarter of that in the tank, but less than one-quarter of that in Moroiso and at the Pier, while the number of brittle star species detected was more than 90% of that at 80 m (Fig. 4; Table 5). Non-metazoan biota was detected in the tank (Proteobacteria), Moroiso surface (Pyramimonadophyceae and Mamiellophyceae), Moroiso bottom (Pyramimonadophyceae), and Pier (Pyramimonadophyceae).

The proportion of OTUs (species)/reads putatively assigned to 1) brittle star was 40.5%/99.4% (Tanks), 22.4%/40.6% (Pier), 18.9%/20.1% (Moroiso surface), 22.5%/16.1% (Moroiso bottom), 90.9%/100% (80 m), and 91.6%/98.3% (deep sea), respectively; 2) those assigned to Echinodermata other than brittle star were 10.8%/0.003% (Tanks), 13.7%/2.1% (Pier), 6.8%/1.3% (Moroiso surface), 6.4%/0.001% (Moroiso bottom), and 0%/0% (80 m), respectively; 3) other metazoans were 43.2%/0.001% (Tanks), 60.3%/56.8% (Pier), 72.4%/78.3% (Moroiso surface), 64.6%/83.5% (Moroiso bottom), and 9%/1.6% (80 m), respectively; 4) non-metazoans were 5.4%/>0.001% (Tank), 6.4%/>0.001% (Moroiso surface), 3.4%/0.001% (Moroiso bottom), and 8.3%/>0.001% (80 m) (Fig. 4; Table 5).

# Discussion

### Usefulness of eDNA metabarcoding with 16SOph primers: 16SOph1 vs 16SOph2

A total of 38 taxa of brittle stars with precise species-level identification (without "Ophiuroidea sp.: RCMBA668.2231" which is registered as an unidentified species in INSD) were obtained from water eDNA from the shallow (including aquarium tank water eDNA) to the deep sea waters around the Misaki Marine Biological Station. The species detected with the primer pairs 16SOph1 and 16SOph2 were respectively: 14/15 species detected in the aquarium water, 10/14 species in the water surface of Moroiso, 11/13 species on the sea bottom of Moroiso, 9/11 species on the pier, and 18/10 species at a depth of 80 m (Table 4; Suppl. material 5: tables S6–S14; Suppl. material 4: figs S11–S15). These results indicate that the 16SOph2 primer has higher detection power for species in shallow water than the 16SOph1 primer. On the other hand, at a depth of 80 m, the number of species detected by 16SOph1 was more than double the number detected by 16SOph2. Based on these results, eDNA metabarcoding was performed using 16SOph1 at 250–270 m depth, which is a sandy and muddy substrate similar to that at 80 m depth. As a result, we obtained 10 brittle star species (including one species of "Ophiuroidea sp.") from these depths that were accurately identified (Table 4; Suppl. material 4: fig. S16).

The difference between the 2 primer sets was clearly shown in the taxa detected other than the brittle stars. In all of the water eDNA samples analyzed in this study, more than 90% of the species and reads detected by 16SOph1 were brittle stars. Considering that the sequences of insects obtained from Moroiso and the deep sea were contaminants, the only other organisms' sequences that were surely picked up were those of 2 nemertean species that were reared together with the brittle stars in the tanks (Fig. 4; Table 5).

On the other hand, among the species detected with 16SOph2, the proportion of brittle stars in shallow water was less than half in all environments, and less than one-quarter in the Moroiso and the Pier. This is the same as the trend in obtained reads: in Pier and Moroiso, the number of reads of brittle stars was less than half. In Tank, however, most of the reads were brittle stars. On the other hand, by detection with16SOph2, mollusca, bryozoans, and crustaceans rather than other echinoderms (asteroids, crinoids, echinoids, and holothuroids) were more abundant in Moroiso and Pier. The proportions of non-metazoans (plants and bacteria) in both 16SOph1 and 16SOph2 were quite small in all environments (Fig. 4; Table 5).

These results suggest that 16SOph1 performs better at detecting mainly brittle stars, while 16SOph2 is able to detect a broader range of marine metazoa. In addition to the detected brittle stars, other species were found to be distributed in the Misaki area, suggesting that this is not a result of contamination. However, since this study focuses on brittle stars, we do not discuss the results of other taxa in detail here.

In addition, at a depth of 80 m, even with 16SOph2, only 1 species of crustacean was detected. One hypothesis to explain this is that there is a very large amount of eDNA from brittle stars at the bottom of the deeper sea area, much more than eDNA from crustaceans. In order to verify this hypothesis, we need to collect water samples in many more deep-sea environments and test other primers in the future.

In the last 10 years, 59 morphologically identified species have been collected from the MMBS (Table 4). As a result of the eDNA metabarcoding analysis in this study, we were able to detect at least 38 of these species, which means that we have succeeded in developing a method for obtaining biodiversity information without collecting specimens or conducting visual observations. There have been few studies on eDNA metabarcoding of deep-sea organisms at depths deeper than 200 m based on comparison with detailed collected specimen data. Therefore, this study provides a method to expand our knowledge of environmental monitoring in the ocean, which contains more than 90% of the Earth's biosphere.

It should also be noted that the genetic distance analysis of sequences obtained by using 16SOph1 and 16SOph2 suggested that *Amphioplus rhadinobrachius*, *Amphiura koreae*, and *Amphiura trachydisca* should be synonyms. The taxonomy of Amphiuridae, to which the three species

belong, has been confusing, and in fact, the recent phylogenomic analysis of brittle stars showed that the genus-level phylogeny including *Amphiura* and *Amphioplus* is polyphyletic (O'Hara et al. 2017). The results of our study are therefore not surprising. We here refrain from synonymising these three species since it is beyond the scope of our study. The species-genus level classification of Amphiuridae should be taxonomically reviewed in the future.

The sequences with 80% < identity < 97% with our custom database 2+3 were obtained for 16SOph1 as follows: 2 species at the tank, 4 species at Moroiso, 3 species at the pier, 20 species at a depth of 80 m, and 6 species at depths of 250–270 m. Because of the ambiguity of identification, we refrain from mentioning these species in this study. However, considering the number of the detected species, it is suggested that there are many brittle stars that have not yet been discovered from Sagami Bay. On the other hand, the sequences with identity less than 93.85% (uncertain identification) were not detected by 16SOph2.

In terms of the total number of reads (and their percentages compared to all detected metazoan taxa): 338,367 (99.9%)/522,016 (99.4%) in the aquarium tank; 109,680 (99.9%)/5,791 (16.1%) in Moroiso (including surface and sea bottom), 86,056 (100%)/11,186 (20.1%) in the Pier, and 182,951 (100%)/755 (98.3%), were detected using 16SOph1 and 16Soph2, respectively (Table 5). Considering these results, it can be seen that although the number of detected reads of Oph16S2 was high in the aquarium tank, the overall trend was for those of Oph16S1 to be higher. The proportions of reads of brittle stars among all metazoans in the tank and at 80 m were almost the same for both primers, but those in Moroiso and the Pier were much higher for 16SOph1 than for 16SOph2 (Table 5). These results clearly showed the characteristics of 16SOph1 targeting the conserved region and 16SOph2 targeting the hyper-variable region. The number of species with certain identification was higher for 16SOph2, but the number of species with uncertain identification was higher for 16SOph1 (Suppl. material 5: tables S6–S14). This may be attributed to the shorter target sequence of the former primer, which could more easily pick up DNA fragmented in environmental water. This is also consistent with the fact that 16SOph1 had a higher number of detected reads. However, the fact that 16SOph2 amplified the DNA sequences of other marine animals other than brittle stars was unexpected. This may be a result of chance. In addition, when phylogenetic trees were constructed, 16SOph2 tended to have longer branches between each monophyletic clade than 16SOph1 and higher bootstrap values supporting the monophyly of each clade (Suppl. material 4: figs S2–S10). Thus, 16SOph1 and 16SOph2 have their own advantages and disadvantages. Therefore, it is expected that more accurate measurement of the marine environment will be possible by using one primer or the other for each environment, or by using both. In the following sections, we would like to discuss the detected species as follows. For the taxonomic notes for the detected species, see Suppl. material 2.

### Taxa detected from eDNA metabarcoding – Aquarium tanks

In this study, we examined the detection power of the developed primers from the aquarium waters where the brittle stars were actually reared (Fig. 2E, F). As a result, 12 (16SOph1) and 13 (16SOph2) taxa were detected by each primer among 15 reared species (Suppl. material 4: fig. S11). Ten species were common to both primers (Suppl. material 5: tables S6, S11). *Ophiactis macrolepidota*1 was detected only by 16SOph1, and *Stegophiura sterea*, *Ophiactis profundi*, and *Ophioplocus japonicus* were detected only by 16SOph2. Of these, *O. japonicus*, for example, is a common species that occurs in the shallow waters around MMBS. However, considering the fact that this species was detected only by 16SOph2 at Moroiso and the Pier, it is clear that there was a difference in the detection trend for each primer.

### Taxa detected by eDNA metabarcoding – Moroiso

Ten (16SOp1) and 14 species (16SOp2) were detected in the surface water of Moroiso (Suppl. material 5: tables S8, S9). Other than *Amphiura sinicola*, the species detected by 16SOph1 were also detected by 16SOph2, and *Ophiuroglypha kinbergi*, *Ophiothela danae*, *Ophiocentrus tokiokai*, and *Ophiactis lymani* were detected only by 16SOph2. Therefore, 16SOph2 was more powerful in detecting the brittle stars in the water surface of Moroiso (Suppl. material 4: figs S12, S13; Suppl. material 5: tables S9, S10).

Eleven species (16SOph1) and 13 species (16SOph2) were detected on the sea bottom of Moroiso (Suppl. material 5: tables S8, S9). Among them, *Ophioplocus japonicus*, *Ophiocentrus tokiokai*, *Ophiactis lymani*, and *Ophiothrix exigua* were detected only by 16SOph2, and *Ophiomaza cacaotica* and *Ophionereis dubia* were detected only by 16SOph1. Also on the sea bottom of Moroiso, 16SOph2 was more powerful in detection of taxa of brittle stars than 16SOph1 (Suppl. material 4: fig. S13; Suppl. material 5: tables S8, S9).

### Taxa detected by eDNA metabarcoding – Pier

Nine taxa (16SOph1) and 11 taxa (16SOph2) were detected from the Pier (Suppl. material 5: tables S10, S11). *Ophiactis lymani*, *Amphichilus trichoides*, and *Macrophiothrix longipeda* were detected only by 16Soph2, and *Ophiomastix mixta* was detected only by 16SOph1. The detection power of 16SOph2 was higher than that of 16SOph1 (Table 4; Suppl. material 4: fig. S14; Suppl. material 5: tables S10, S11).

### Taxa detected from eDNA metabarcoding – 80 m

At a depth of 80 m, 18 species (16SOph1) and 10 species (16SOph2) of brittle stars were detected. In this area, species detected by 16SOph2 were generally also detected by 16SOph1, except for *Ophiothrix nereidina*, which was only

detected by 16SOph2. Therefore, the detection power of 16SOph1 was higher than that of 16SOph2 in this area (Suppl. material 4: fig. S15; Suppl. material 5: tables S12, S13).

### Taxa detected from eDNA metabarcoding – Deep sea (only 16SOph1)

Nine species were detected from 250–270 m depth (Suppl. material 5: table S14). This sampling area is also an area frequently surveyed by dredges of the laboratory research boat, and at least 23 species (considering synonyms) have been recorded there so far. In this study, *Ophiothrix nereidina* and *Ophiosparte gigas* were newly detected by eDNA metabarcoding (Suppl. material 4: fig. S16; Suppl. material 5: table S14).

### Future challenges – Optimization of eDNA metabarcoding

False negatives (not detected even though present) and false positives (detected even though absent) are important issues for eDNA metabarcoding because they can lead to underestimation or overestimation of biodiversity (e.g. Komai et al. 2019). The results of the present analysis showed that many of the species inhabiting the shallow waters were actually detected (Suppl. material 5: tables S6–S14). On the other hand, many of the brittle star species recorded from 80 m and 250–270 m were not detected in the present study; and conversely, many sequences of species that are supposed not to exist or not to be collected in these area were detected (Table 4; Suppl. material 5: tables S9, S13, S14; Suppl. material 2).

Factors causing false negatives include 1) DNA of the target species was not collected during sampling, and 2) DNA of the target species was not amplified well by PCR (e.g. Komai et al. 2019). Regarding the first factor, the difficulty of collecting water using a water sampler can be mentioned. Because the water sampler is lowered to the seafloor on a single string, the deeper the water depth, the greater the amplitude of the water sampler near the seafloor. Therefore, in deep water, the slight rocking of the boat is greatly transmitted to the water sampler on the seafloor, and consequently the sampler tends to move away from the seafloor. In this study, we did not observe the actual water sampling, so we do not know if we were able to drop the water sampler just above the seafloor. In the future, we expect to be able to collect more eDNA by collecting a large amount of water as close to the seafloor as possible using an ROV and larger water sampler.

In order to solve the second problem, it is necessary to develop optimal experimental conditions using 16SOph primers. In the future, increasing the number of PCR replicates and using multiple annealing temperatures, as well as the revision of primer sequences, will allow us to detect more target eDNA (Komai et al. 2019).

Finally, a third factor, DNA shedding, must be taken into account. In seawater, DNA from all organisms is not always present. An organism's DNA is shed at some level, and the rate of shedding should differ among taxa. Therefore, no matter how efficient the water sampling is or how suitable the primers are, some organisms that live in special environments and have specific ecologies will remain undetectable. In the future, it will still be important to supplement data by collecting organisms that inhabit the study area, rather than relying solely on environmental DNA for monitoring.

One of the most important points for accurate assessment of biodiversity by eDNA metabarcoding is to improve the reference sequence database, which is essential for taxonomic assignment. In this study, we created a custom database for the preparation of primers by adding our own sequence data to the INSD database. Currently, 350 species of brittle stars are known from Japanese waters (Okanishi 2016; Okanishi and Fujita 2018a, b, c; Okanishi et al. 2019; Okanishi et al. 2020a, b; Okanishi and Kohtsuka 2021), which is about 17% of the currently known brittle star species (32 families, ca. 2100 species; Stöhr et al. 2012). In addition, there are many undescribed cryptic species or species complexes, such as *Amphipholis squamata* (Boissin et al. 2008) and *Asteronyx loveni* (Okanishi et al. 2018), and *Ophiactis savignyi* (Roy and Sponer 2002), and such species were also detected in this study (e.g. *Ophiothrix panchyenchyta*1 and 2; Table 4; Suppl. material 2). In order to solve the cryptic species problem, a thorough quantitative morphometrical approach and re-examination of type specimens with observation of internal ossicle morphology are required, along with DNA phylogenetic analysis as in a study by dos Santos Alitto et al. (2019).

Overrepresentation of a few frequently sequenced species in INSD is also an issue that should be noted. In this study, more than 95% of the sequences that were identified with certainty were compared to sequences that we obtained in this study or from a previous study (e.g. Okanishi and Fujita 2013). Therefore, we do not anticipate that our study was affected by the overrepresented sequences accumulated on the INSD due to primer bias. However, in future studies, it will be necessary to take into account the effects of such sequences and remove the overrepresented sequences appropriately.

In this study, we sequenced the partial 16S rRNA genes of 59 brittle star species (three of these species are suspected to be synonyms; Suppl. material 2) obtained from around the study area, among which 18 species (31.5%) did not match eDNA (Table 4). Brittle stars in Sagami Bay have been studied for more than 100 years, starting with Matsumoto's research (e.g. Matsumoto 1917; Irimura 1982; Fujita et al. 2006). However, the fact that many unknown sequences (comprising at least 33 species) were obtained by eDNA metabarcoding indicates that the taxonomic study of brittle stars is still incomplete even in this region.

There are many taxa, such as the brittle star, that have not received much attention so far, but are, in fact, rich in DNA and have great potential as research targets for environmental monitoring. Until now, much data on COI sequences has been accumulated in animals as so-called "DNA barcoding regions" (Hebert et al. 2003). In recent years, however, it

has been recognized that mitochondrial rRNA sequences are suitable for eDNA monitoring in a variety of organisms (e.g., Miya et al. 2015; Komai et al. 2019). For example, in fish, information on the mitochondrial genome was originally accumulated for each species. Therefore, it was possible to convert the data into reference sequence data (Iwasaki et al. 2013; Miya et al. 2015). For other taxa, however, information on the entire mitochondrial genome has not yet been accumulated, and more time will be needed to construct a reference sequence database for eDNA monitoring.

In addition to the conventional COI-based DNA barcoding project, it will be necessary to determine the data of mitochondrial genes, including 16S rRNA, for each species to optimize the usefulness of eDNA metabarcoding analysis. This will require a significant taxonomic update by taxonomists.

In this study, we constructed a database based on specimens actually collected and organized by taxonomists, and laid the foundation for an eDNA metabarcoding method for brittle star in the deep sea. The oceans occupy most of the Earth's biosphere, and marine animals account for most of the Earth's animal biomass (Bar-On et al. 2018). Therefore, in order to accurately measure the biodiversity of the Earth, it is essential to accurately understand the diversity of marine organisms. This study is expected to be a pioneering study in deep-sea research, where eDNA metabarcoding has rarely been performed due to the difficulty of access, despite the fact that the volume of the deep sea is the largest in the ocean.

## Conclusion

In this study, by using 2 newly developed sets of PCR primers for metabarcoding environmental DNA (eDNA) for brittle stars, we performed eDNA metabarcoding from natural seawater collected at Misaki, Miura, Kanagawa, the Pacific coast of central Japan, covering shallow (2 m) to deep sea (> 200 m) waters, and aquarium tanks. Comparison of the obtained eDNA sequences with our new custom database of 16S rRNA sequences of brittle stars, 37 (including cryptic species) and 26 brittle star species were detected by 16SOph1 and 16SOph2 with sequence identities of > 97.14% and of > 93.85%, respectively.

The proportion of species other than brittle stars detected with 16SOph1 was less than 10% of the total number of species, while that with 16SOph2 was less than half in shallow water. On the other hand, the proportion at 80 m was less than 10% with both of the primer sets. These evidences suggest that 16SOph1 is a primer set specific to the brittle star and 16SOph2 is suitable for a variety of marine metazoans.

## Data accessibility

16SOph1, 16SOph2 sequences from the 59 brittle stars are available from INSD (Table 4). Raw reads from the MiSeq sequencing and custom reference fasta data are available from figshare (DOI https://doi.org/10.6084/m9.figshare.22140200.v1 and https://doi.org/10.6084/m9.figshare.22139909.v1).

## Author contributions

MO: planned the study, acquired funding, performed the sampling of environment waters, performed identification of all sampled specimens and experiments, analyzed the data, and wrote the manuscript with creation of all tables and figures.

HK: collected all examined specimens, performed sampling of waters, and contributed to the manuscript preparation with creation of a part of figures.

QW: contributed to the data analysis, especially regarding newly developed primers, and to the manuscript preparation.

JS: contributed to the data analysis, especially regarding amplifying 16S rRNA regions from specimens, and to the manuscript preparation.

NS: contributed to the data analysis, especially regarding newly developed primers, and to extraction of brittle star sequences from raw data, metabarcoding of eDNA, and the manuscript preparation.

TT: contributed to the data analysis, especially regarding newly developed primers, metabarcoding of environmental DNA, and the manuscript preparation.

TN: acquired funding, contributed to the manuscript preparation.

TM: planned the study, acquired funding, and contributed to preparation of the manuscript.

## Conflict of interest

TM is an inventor of the patent for the use of BAC for eDNA preservation. The other authors have declared that no competing interests exist.

## Supporting agencies

JSPS - Japan Society for the Promotion of Science.

Ministry of Agriculture, Forestry, and Fisheries of Japan; JST/JICA: Science and Technology Research Partnership for Sustainable Development; Estonian Ministry of Education and Research; Mobilitas Pluss.

## Acknowledgements

We are most grateful to Dr. Elizabeth Nakajima (freelance scientific English editor) for her careful and critical readings of the manuscript and providing constructive comments. We also thank Mamoru Sekifuji and Michiyo Kawabata of Misaki Marine Biological Station, for their kind assistance during sampling by dredge and water sampling bottle. This work was partly supported by KAKEN-

HI Grant Number 21K05632 and 25440226 to MO and by the Environment Research and Technology Development Fund (JPMEERF20S20704) of the Environmental Restoration and Conservation Agency, Japan to TM and TN.

# References


Andruszkiewicz EA, Starks HA, Chavez FP, Sassoubre LM, Block BA, Boehm AB (2017) Biomonitoring of marine vertebrates in Monterey Bay using eDNA metabarcoding. PLoS ONE 12(4): 1–20. https://doi.org/10.1371/journal.pone.0176343

Bar-On YM, Phillips R, Milo R (2018) The biomass distribution on Earth. Proceedings of the National Academy of Sciences of the United States of America 115(25): 6506–6511. https://doi.org/10.1073/pnas.1711842115

Bohmann K, Evans A, Gilbert MTP, Carvalho GR, Creer S, Knapp M, Yu DW, de Bruyn M (2014) Environmental DNA for wildlife biology and biodiversity monitoring. Trends in Ecology & Evolution 29(6): 358–367. https://doi.org/10.1016/j.tree.2014.04.003

Boissin E, Féral JP, Chenuil A (2008) Defining reproductively isolated units in a cryptic and syntopic species complex using mitochondrial and nuclear markers: The brooding brittle star, Amphipholis squamata (Ophiuroidea). Molecular Ecology 17(7): 1732–1744. https://doi.org/10.1111/j.1365-294X.2007.03652.x

Cho W, Shank T (2010) Incongruent patterns of genetic connectivity among four ophiuroid species on North Atlantic seamounts. Marine Ecology (Berlin) 31(s1): 121–143. https://doi.org/10.1111/j.1439-0485.2010.00395.x

Coissac E, Riaz T, Puillandre N (2012) Bioinformatic challenges for DNA metabarcoding of plants and animals. Molecular Ecology 21(8): 1834–1847. https://doi.org/10.1111/j.1365-294X.2012.05550.x

Deagle BE, Jarman SN, Coissac E, Pompanon F, Taberlet P (2014) DNA metabarcoding and the cytochrome c oxidase subunit I marker: Not a perfect match. Biology Letters 10(9): 2–5. https://doi.org/10.1098/rsbl.2014.0562

Díaz-Ferguson EE, Moyer GR (2014) History, applications, methodological issues and perspectives for the use of environmental DNA (eDNA) in marine and freshwater environments. Revista de Biología Tropical 62(4): 1273–1284. https://doi.org/10.15517/rbt.v62i4.13231

dos Santos Alitto RA, Amaral ACZ, de Oliveira LD, Serrano H, Seger KR, Guilherme PDB, Di Domenico M, Christensen AB, Lourenço LB, Tavares M, Borges M (2019) Atlantic west Ophiothrix spp. In the scope of integrative taxonomy: Confirming the existence of Ophiothrix trindadensis Tommasi, 1970. PLoS ONE 14(1): 1–28. https://doi.org/10.1371/journal.pone.0210331

Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics (Oxford, England) 26(19): 2460–2461. https://doi.org/10.1093/bioinformatics/btq461

Fujikura K, Okutani T, Maruyama T (2012) Deep-sea Life. Biological observations using research submersibles. 2nd ed. Fujikura K, Okutani T, Maruyama T (Eds). Tokai University Press, Tokyo, 487 pp.

Fujita T, Ishida Y, Irimura S (2006) Preliminary report of Ophiacanthidae (Echinodermata, Ophiuroidea) collected from the Sagami Sea, Central Japan. Memoirs of the National Science Museum 41: 289–303.

Gage JD, Tyler PA (1991) Deep-Sea Biology: A Natural History of Organisms at the Deep-Sea Floor. Campridge University Press, Cambridge, UK, 504 pp. https://doi.org/10.1017/CBO9781139163637

Gielings R, Fais MFD, Creer S, Costa FO, Renema W, Macher J-N (2021) DNA metabarcoding methods for the study of marine benthic meiofauna: A review. Frontiers in Marine Science 8: 730063. [13 pp] https://doi.org/10.3389/fmars.2021.730063

Glynn PW, Alitto R, Dominguez J, Christensen AB, Gillette P, Martinez N, Riegl BM, Dettloff K (2020) A tropical eastern Pacific invasive brittle star species (Echinodermata: Ophiuroidea) reaches southeastern Florida. In: Bernhard MR (Ed.) Advances in Marine Biology vol 87, 1st ed. Academic Press, Massachusetts, 443–472. https://doi.org/10.1016/bs.amb.2020.08.010

Gray MW, Sankoff D, Cedergren RJ (1984) On the evolutionary descent of organisms and organelles: A global phylogeny based on a highly conserved structural core in small subunit ribosomal RNA. Nucleic Acids Research 12(14): 5837–5852. https://doi.org/10.1093/nar/12.14.5837

Hebert PDN, Cywinska A, Ball SL, DeWaard JR (2003) Biological identifications through DNA barcodes. Proceedings. Biological Sciences 270(1512): 313–321. https://doi.org/10.1098/rspb.2002.2218

Hunter R, Halanych K (2008) Evaluating connectivity in the brooding brittle star *Astrotoma agassizii* across the Drake Passage in the Southern Ocean. The Journal of Heredity 99(2): 137–148. https://doi.org/10.1093/jhered/esm119

Irimura S (1982) The brittle-stars of Sagami Bay, collected by His Majesty the Emperor of Japan. Biological Laboratory, Imperial Household, Japan, 95 pp.

Iwasaki W, Fukunaga T, Isagozawa R, Yamada K, Maeda Y, Satoh TP, Sado T, Mabuchi K, Takeshima H, Miya M, Nishida M (2013) Mitofish and mitoannotator: A mitochondrial genome database of fish with an accurate and automatic annotation pipeline. Molecular Biology and Evolution 30(11): 2531–2540. https://doi.org/10.1093/molbev/mst141

Kelly RP, Port JA, Yamahara KM, Crowder LB (2014) Using environmental DNA to census marine fishes in a large mesocosm. PLoS ONE 9(1): e86175. https://doi.org/10.1371/journal.pone.0086175

Komai T, Gotoh RO, Sado T, Miya M (2019) Development of a new set of PCR primers for eDNA metabarcoding decapod crustaceans. Metabarcoding and Metagenomics 3: 1–19. https://doi.org/10.3897/mbmg.3.33835

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular Biology and Evolution 33(7): 1870–1874. https://doi.org/10.1093/molbev/msw054

Madduppa H, Cahyani NKD, Anggoro AW, Subhan B, Jefri E, Sani LMI, Arafat D, Akbar N, Bengen DG (2021) eDNA metabarcoding illuminates species diversity and composition of three phyla (chordata, mollusca and echinodermata) across Indonesian coral reefs. Biodiversity and Conservation 30(11): 3087–3114. https://doi.org/10.1007/s10531-021-02237-0

Matsumoto H (1917) A monograph of Japanese Ophiuroidea, arranged according to a new classification. Journal of the College of Science, Imperial University, Tokyo 38: 1–408.

McClenaghan B, Fahner N, Cote D, Chawarski J, McCarthy A, Rajabi H, Singer G, Hajibabaei M (2020) Harnessing the power of eDNA metabarcoding for the detection of deep-sea fishes. PLoS ONE 15(11): 1–19. https://doi.org/10.1371/journal.pone.0236540

Miya M, Sato Y, Fukunaga T, Sado T, Poulsen JY, Sato K, Minamoto T, Yamamoto S, Yamanaka H, Araki H, Kondoh M, Iwasaki W (2015) MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: Detection of more than 230 subtropical

marine species. Royal Society Open Science 2(7): 15088. https://doi.org/10.1098/rsos.150088

O'Hara TD, Hugall AF, Thuy B, Stöhr S, Martynov AV (2017) Restructuring higher taxonomy using broad-scale phylogenomics: The living Ophiuroidea. Molecular Phylogenetics and Evolution 107: 415–430. [Epub2016Dec8] https://doi.org/10.1016/j.ympev.2016.12.006

Okanishi M (2016) Ophiuroidea (Echinodermata): Systematics and Japanese Fauna. In: Motokawa M, Kajihara H (Eds) Species Diversity of Animals in Japan. Springer Japan, Tokyo, 651–678. https://doi.org/10.1007/978-4-431-56432-4_25

Okanishi M, Fujita T (2013) Molecular phylogeny based on increased number of species and genes revealed more robust family-level systematics of the order Euryalida (Echinodermata: Ophiuroidea). Molecular Phylogenetics and Evolution 69(3): 566–580. https://doi.org/10.1016/j.ympev.2013.07.021

Okanishi M, Fujita Y (2018a) A new species of Ophioconis (Echinodermata Ophiuroidea) from Ryukyu Islands, southwestern Japan. Proceedings of the Biological Society of Washington 131(1): 163–174. https://doi.org/10.2988/18-00001

Okanishi M, Fujita T (2018b) A taxonomic review of the genus *Astrodendrum* (Echinodermata, Ophiuroidea, Euryalida, Gorgonocephalidae) with description of a new species from Japan. Zootaxa 4392(2): 289. https://doi.org/10.11646/zootaxa.4392.2.4

Okanishi M, Fujita T (2018c) Description of a new subfamily, Astrocloninae (Ophiuroidea: Euryalida: Gorgonocephalidae), based on molecular phylogeny and morphological observations. Zoological Science 35(2): 179–187. https://doi.org/10.2108/zs170090

Okanishi M, Kohtsuka H (2021) Description of a new brooding species of *Ophiodelos* (Echinodermata: Ophiuroidea) from Japan. Zoological Science 38(4): 352–358. https://doi.org/10.2108/zs200101

Okanishi M, Sentoku A, Martynov A, Fujita T (2018) A new cryptic species of *Asteronyx* Müller and Troschel, 1842 (Echinodermata: Ophiuroidea), based on molecular phylogeny and morphology, from off Pacific Coast of Japan. Zoologischer Anzeiger 274: 14–33. https://doi.org/10.1016/j.jcz.2018.03.001

Okanishi M, Oba Y, Fujita Y (2019) Brittle stars from a submarine cave of Christmas Island, northwestern Australia, with description of a new bioluminescent species *Ophiopsila xmasilluminans* (Echinodermata: Ophiuroidea) and notes on its behaviour. The Raffles Bulletin of Zoology 67: 421–439. https://doi.org/10.26107/RBZ-2019-0034

Okanishi M, Kohtsuka H, Fujita T (2020a) A taxonomic review of the genus *Astrocladus* (Echinodermata, Ophiuroidea, Euryalida, Gorgonocephalidae) from Japanese coastal waters. PeerJ 8: 9836. https://doi.org/10.7717/peerj.9836

Okanishi M, Kato M, Watanabe H, Chong C, Fujita T (2020b) Large populations of two new species of *Ophiambix* (Echinodermata, Ophiuroidea) discovered on Japanese hot vents and cold seeps. The Raffles Bulletin of Zoology 68: 196–213. https://lkcnhm.nus.edu.sg/wp-content/uploads/sites/10/app/uploads/2020/01/RBZ-2020-0017.pdf

Palumbi SR (1996) Nucleic Acids II: The Polymerase Chain Reaction. Molecular Systematics 2: 43.

Rees HC, Maddison BC, Middleditch DJ, Patmore JRM, Gough KC (2014) The detection of aquatic animal species using environmental DNA - a review of eDNA as a survey tool in ecology. Journal of Applied Ecology 51(5): 1450–1459. https://doi.org/10.1111/1365-2664.12306

Riaz T, Shehzad W, Viari A, Pompanon F, Taberlet P, Coissac E (2011) EcoPrimers: Inference of new DNA barcode markers from whole genome sequence analysis. Nucleic Acids Research 39(21): 1–11. https://doi.org/10.1093/nar/gkr732

Roy MS, Sponer R (2002) Evidence of a human-mediated invasion of the tropical western Atlantic by the "world's most common brittlestar.". Proceedings of the Royal Society B: Biological Sciences 269(1495): 1017–1023. https://doi.org/10.1098/rspb.2002.1977

Schnell IB, Bohmann K, Gilbert MTP (2015) Tag jumps illuminated – reducing sequence-to-sample misidentifications in metabarcoding studies. Molecular Ecology Resources 15: 1289–1303. https://doi.org/10.1111/1755-0998.12402

Stöhr S, O'Hara TD, Thuy B (2012) Global diversity of brittle stars (Echinodermata: Ophiuroidea). PLoS ONE 7(3): e31940. https://doi.org/10.1371/journal.pone.0031940

Taberlet P, Coissac E, Hajibabaei M, Rieseberg LH (2012) Environmental DNA. Molecular Ecology 21(8): 1789–1793. https://doi.org/10.1111/j.1365-294X.2012.05542.x

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22(22): 4673–4680. https://doi.org/10.1093/nar/22.22.4673

Thomsen PF, Kielgast J, Iversen LL, Møller PR, Rasmussen M, Willerslev E (2012) Detection of a diverse marine fish fauna using environmental DNA from seawater samples. PLoS ONE 7(8): 1–9. https://doi.org/10.1371/journal.pone.0041732

Tsuji S, Shibata N, Sawada H, Ushio M (2020) Quantitative evaluation of intraspecific genetic diversity in a natural fish population using environmental DNA analysis. Molecular Ecology Resources 20(5): 1323–1332. https://doi.org/10.1111/1755-0998.13200

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012) Primer3-new capabilities and interfaces. Nucleic Acids Research 40(15): 1–12. https://doi.org/10.1093/nar/gks596

Ushio M, Fukuda H, Inoue T, Makoto K, Kishida O, Sato K, Murata K, Nikaido M, Sado T, Sato Y, Takeshita M, Iwasaki W, Yamanaka H, Kondoh M, Miya M (2017) Environmental DNA enables detection of terrestrial mammals from forest pond water. Molecular Ecology Resources 17(6): e63–e75. https://doi.org/10.1111/1755-0998.12690

Ushio M, Murata K, Sado T, Nishiumi I, Takeshita M, Iwasaki W, Miya M (2018) Demonstration of the potential of environmental DNA as a tool for the detection of avian species. Scientific Reports 8(1): 1–10. https://doi.org/10.1038/s41598-018-22817-5

Valentini A, Taberlet P, Miaud C, Civade R, Herder J, Thomsen PF, Bellemain E, Besnard A, Coissac E, Boyer F, Gaboriaud C, Jean P, Poulet N, Roset N, Copp GH, Geniez P, Pont D, Argillier C, Baudoin JM, Peroux T, Crivelli AJ, Olivier A, Acqueberge M, Le Brun M, Møller PR, Willerslev E, Dejean T (2016) Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. Molecular Ecology 25(4): 929–942. https://doi.org/10.1111/mec.13428

Wu Q, Kawano K, Uehara Y, Okuda N, Hongo M, Tsuji S, Yamanaka H, Minamoto T (2018) Environmental DNA reveals nonmigratory individuals of Palaemon paucidens overwintering in Lake Biwa shallow waters. Freshwater Science 37(2): 307–314. https://doi.org/10.1086/697542

Yamanaka H, Minamoto T, Matsuura J, Sakurai S, Tsuji S, Motozawa H, Hongo M, Sogo Y, Kakimi N, Teramura I, Sugita M, Baba M, Kondo A (2017) A simple method for preserving environmental DNA in water samples at ambient temperature by addition of cationic surfactant. Limnology 18(2): 233–241. https://doi.org/10.1007/s10201-016-0508-5

**Supplementary material 1**

**A fasta file of 1,340 mitochondrial 16S rRNA gene sequences**

Author: Masanori Okanishi, Hisanori Kohtsuka, Qianqian Wu, Jumpei Shinji, Naoki Shibata, Takashi Tamada, Tomoyuki Nakano, Toshifumi Minamoto

Data type: fasta file

Explanation note: A fasta file of 1,340 mitochondrial 16S rRNA gene sequences from 33 known brittle star families which were downloaded from INSDC on 17 December 2021.

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/mbmg.7.94298.suppl1

**Supplementary material 2**

**Taxonomic notes for detected taxa**

Author: Masanori Okanishi, Hisanori Kohtsuka, Qianqian Wu, Jumpei Shinji, Naoki Shibata, Takashi Tamada, Tomoyuki Nakano, Toshifumi Minamoto

Data type: MS Word document

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/mbmg.7.94298.suppl2

**Supplementary material 3**

**A specimen list of brittle star species collected from Sagami Bay in the last 10 years**

Author: Masanori Okanishi, Hisanori Kohtsuka, Qianqian Wu, Jumpei Shinji, Naoki Shibata, Takashi Tamada, Tomoyuki Nakano, Toshifumi Minamoto

Data type: MS Excel document

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/mbmg.7.94298.suppl3

**Supplementary material 4**

**Supplementary images S1–S16**

Author: Masanori Okanishi, Hisanori Kohtsuka, Qianqian Wu, Jumpei Shinji, Naoki Shibata, Takashi Tamada, Tomoyuki Nakano, Toshifumi Minamoto

Data type: figures (JPG images in ZIP. Archive)

Explanation note: **fig. S1**: Schematic diagram of the positional relationship of the primers used in this study on a partial region of 16S rRNA gene of brittle stars. **fig. S2**: Maximum likelihood tree of brittle star sequences amplified from environmental water in aquarium tanks of MMBS by the 16SOph1 primer; based on T92 + G nucleotide substitution model. Bootstrap support values (100 replications) are shown on the branches. Species names with certain identification are indicated by bold. The numbers after species name on each OTU indicate sequence similarity (%), length of the analyzed sequence, and the number of mismatch nucleotides with a best hit reference sequence. **fig. S3**: Maximum likelihood tree of brittle star sequences amplified from environmental water in aquarium tanks of MMBS by the 16SOph2 primer; based on T92 + G + I nucleotide substitution model. Bootstrap support values (100 replications) are shown on the branches. Species names with certain identification are indicated by bold. The numbers after species name on each OTU indicate sequence similarity (%), length of the analyzed sequence, and the number of mismatch nucleotides with a best hit reference sequence. **fig. S4**: Maximum likelihood tree of brittle star sequences amplified from environmental water in Moroiso (including both surface and sea bottom) by the 16SOph1 primer; based on the T92 + G nucleotide substitution model. Bootstrap support values (100 replications) are shown on the branches. Species names with certain identification are indicated by bold. The numbers after species name on each OTU indicate sequence similarity (%), length of the analyzed sequence, and the number of mismatch nucleotides with a best hit reference sequence. **fig. S5**: Maximum likelihood tree of brittle star sequences amplified from environmental water in Moroiso (including both surface and sea bottom) by the 16SOph2 primer; based on T92 + G nucleotide substitution model. Bootstrap support values (100 replications) are shown on the branches. Species names with certain identification are indicated by bold. The numbers after species name on each OTU indicate sequence similarity (%), length of the analyzed sequence, and the number of mismatch nucleotides with a best hit reference sequence. **fig. S6**: Maximum likelihood tree of brittle star sequences amplified from environmental water in the Pier at MMBS by the 16SOph1 primer; based on T92 + G nucleotide substitution model. Bootstrap support values (100 replications) are shown on the branches. Species names with certain identification are indicated by bold. The numbers after species name on each OTU indicate sequence similarity (%), length of the analyzed sequence, and the number of mismatch nucleotides with a best hit reference sequence. **fig. S7**: Maximum likelihood tree of brittle star sequences amplified from environmental water in the Pier at MMBS by the 16SOph2 primer; based on T92 + G nucleotide substitution model. Bootstrap support values (100 replications) are shown on the branches. Species names with certain identification are indicated by bold. The numbers after species name on each OTU indicate sequence similarity (%), length of the analyzed sequence, and the number of mismatch nucleotides with a best hit reference sequence. **fig. S8**: Maximum likelihood tree of brittle star sequences amplified from environmental water at a depth of 80 m off MMBS by the 16SOph1 primer; based on T92 + G nucleotide substitution model. Bootstrap support values (100 replications) are shown on the branches. Species names with certain identification are indicated as bold. The numbers after species name on each OTU indicate sequence similarity (%), length of the analyzed sequence, and the number of mismatch nucleotides with a best hit reference sequence. **fig. 9**: Maximum likelihood tree of brittle star sequences amplified from environmental water at a depth of 80 m off MMBS by the 16SOph2 primer; based on T92 + G nucleotide substitution model. Bootstrap support values (100 replications) are shown on the branches. Species names with certain identification are indicated as bold. The numbers after species name on each OTU indicate sequence similarity (%), length of the analyzed sequence, and the number of mismatch nucleotides with a best hit reference sequence. **fig. 10**: Maximum likelihood tree of brittle star sequences amplified from environmental water at the depths of 250 m

and 270 m off MMBS by the 16SOph1 primer; based on T92 + G + I nucleotide substitution model. Bootstrap support values (100 replications) are shown on the branches. Species names with certain identification are indicated as bold. The numbers after species name on each OTU indicate sequence similarity (%), length of the analyzed sequence, and the number of mismatch nucleotides with a best hit reference sequence. **fig. S11**: Venn diagram showing the distribution of detected brittle star species from environmental DNA in rearing water of aquarium tank at MMBS. **fig. S12**: Venn diagram showing the distribution of detected brittle star species from environmental DNA in surface water of Moroiso, Miura, Kanagawa, Japan. **fig. S13**: Venn diagram showing the distribution of detected brittle star species from environmental DNA in bottom water of Moroiso, Miura, Kanagawa, Japan. **fig. S14**: Venn diagram showing the distribution of detected brittle star species from environmental DNA in the pier of MMBS. **fig. S15**: Venn diagram showing the distribution of detected brittle star species from environmental DNA of 80 m depth, off MMBS. **fig. S16**: Venn diagram showing the distribution of detected brittle star species from environmental DNA of 250 and 270 m depth, off MMBS. Occurrence records reflect synonyms.

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/mbmg.7.94298.suppl4

**Supplementary material 5**
**Additional data**

Author: Masanori Okanishi, Hisanori Kohtsuka, Qianqian Wu, Jumpei Shinji, Naoki Shibata, Takashi Tamada, Tomoyuki Nakano, Toshifumi Minamoto

Data type: tables (xlsx. files in ZIP. Archive)

Explanation note: **table S1**: The volume of the tanks from which environmental water was collected in this study, and the species with individual numbers (n) kept in the tanks. *Ophiomastix mixta* and *Ophiothela danae* were so numerous that accurate counts unavailable. Species names that were detected in eDNA of the tank are numbered with the corresponding primer number (1: Ophi16S1; 2: Oph16S2). **table S2**: Pairwise genetic distances between the sequences of 16SOph2 region in custom database 2. **table S3**: Pairwise genetic distances between the sequences of 16SOph1 region in custom database 2. **table S4**: Pairwise genetic distances between the sequences of 16SOph1 region in custom database 3. **table S5**: Pairwise genetic distances between the sequences of 16SOph2 region in custom database 3. **table S6**: A list of ophiuroid species detected by 16Soph1 from aquarium tank at MMBS. Number of reads, degree of sequence identity, length of marker sequences (bp), number of sequences, occurrence record of the study area (the records of sequences with 97.14% > identity > 80% were marked with no data, "-"), and accession numbers are summarised. **table S7**: A list of ophiuroid species detected by 16Soph1 from Mroroiso. Number of reads, degree of sequence identity, length of marker sequences (bp), number of sequences, occurrence record of the study area (the records of sequences with 97.14% > identity > 80% were marked with no data, "-"), and accession numbers are summarised. The numbers in parentheses indicate the number of sequences for which reads have been obtained. **table S8**: A list of ophiuroid species detected by 16Soph1 from the Pier. Number of reads, degree of sequence identity, length of marker sequences (bp), number of sequences, occurrence record of the study area (the records of sequences with 97.14% > identity > 80% were marked with no data, ""-""), and accession numbers are summarised. **table S9**: A list of ophiuroid species detected by 16SOph1 at a depth of 80 m off MMBS. Number of reads, degree of sequence identity, length of marker sequences (bp), number of sequences, occurrence record of the study area (the records of sequences with 97.14% > identity > 80% were marked with no data, "-"), and accession numbers are summarised. **table S10**: A list of ophiuroid species detected by 16Soph1 at the depth of 250 m and 270 m off MMBS. Number of reads, degree of sequence identity, length of marker sequences (bp), number of sequences, occurrence record of the study area (the records of sequences with 97.14% > identity > 80% were marked with no data, "-"), and accession numbers are summarised. **table S11:** A list of ophiuroid species detected by 16Soph2 from aquarium tank at MMBS. Number of reads, degree of sequence identity, length of marker sequences (bp), number of sequences, occurrence record of the study area, and accession numbers are summarised. **table S12**: A list of ophiuroid species detected by 16SOph2 from Moroiso. Number of reads, degree of sequence identity, length of marker sequences (bp), number of sequences, occurrence record of the study area, and accession numbers are summarised. The numbers in parentheses indicate the number of sequences for which reads have been obtained. **table S13**: A list of ophiuroid species detected by 16Soph2 from Pier of MMBS. Number of reads, degree of sequence identity, length of marker sequences (bp), number of sequences, occurrence record of the study area, and accession numbers are summarised. **table S14**: A list of ophiuroid species detected by 16SOp2 at a depth of 80 off MMBS. Number of reads, degree of sequence identity, length of marker sequences (bp), number of sequences, occurrence record of the study area, and accession numbers are summarised.

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/mbmg.7.94298.suppl5